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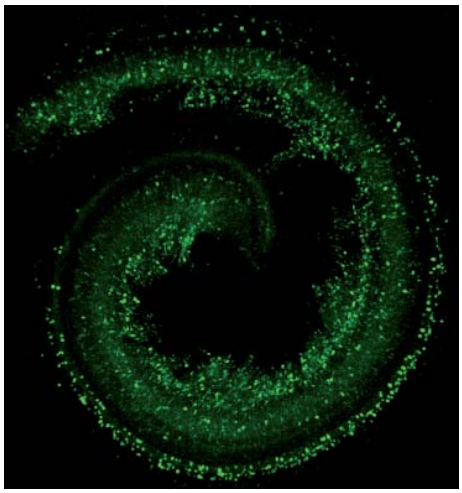
HEIDI LOPONEN

Restrictions in the Proliferative Potential of Inner Ear Hair Cells and Supporting Cells



INSTITUTE OF BIOTECHNOLOGY AND
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RESTRICTIONS IN THE PROLIFERATIVE POTENTIAL OF INNER EAR HAIR CELLS AND SUPPORTING CELLS

Heidi Loponen

Institute of Biotechnology
Research Program in Developmental Biology
and
Division of Genetics
Department of Biosciences
Faculty of Biological and Environmental Sciences
and
Viikki Doctoral Programme in Molecular Biosciences

University of Helsinki

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Supervised by: Docent Ulla Pirvola
Institute of Biotechnology
University of Helsinki
Helsinki, Finland

Reviewed by: Docent Kirmo Wartiovaara
Biomedicum Stem Cell Center
University of Helsinki
Helsinki, Finland
and
Docent Henri Huttunen
Neuroscience Center
University of Helsinki
Helsinki, Finland

Opponent: Dr. Thomas Schimmang
Institute for Biology and Molecular Genetics
University of Valladolid
Valladolid, Spain

Custos: Professor Juha Partanen
Department of Biosciences
University of Helsinki
Helsinki, Finland

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Cover image: A wholemount preparation from a p19Ink4d/p21Cip1 double mutant mouse cochlea shows that normally postmitotic hair cells, located at the outermost region of the cochlear duct, re-enter the cell cycle (are positive for Ki-67, green). See details in papers I and II.

The important thing in science is not so much to obtain new facts as to discover new ways of thinking about them.
~William Lawrence Bragg

Table of Contents

List of Original Publications

This thesis is based on the following original articles (referred in the text by their Roman numerals).

I **Laine H**, Doetzlhofer A, Mantela J, Ylikoski J, Laiho M, Roussel M.F, Segil N, Pirvola U. (2007) p19 (Ink4d) and p21 (Cip1) collaborate to maintain the postmitotic state of auditory hair cells, their codeletion leading to DNA damage and p53-mediated apoptosis. *J Neurosci.* 27: 1434–1444.

II **Laine H**, Sulg M, Kirjavainen A, Pirvola U. (2010) Cell cycle regulation in the inner ear sensory epithelia: role of cyclin D1 and cyclin-dependent kinase inhibitors. *Dev Biol.* 337: 134-146.

III **Loponen H***, Ylikoski J, Albrecht J. H, Pirvola U. (2011) Restrictions in cell cycle progression of adult vestibular supporting cells in response to ectopic cyclin D1 expression. *PLoS One* 6: e27360.

* Loponen formerly Laine

Abbreviations

Ad	Adenovirus
ATM	Ataxia telangiectasia mutated
Atoh1	Atonal homolog 1 against decapentaplegic (MAD) gene
ATR	Ataxia telangiectasia and rad3-related
bHLH	Basic helix-loop-helix (a protein structural motif)
BrdU	5-bromo-2'-deoxyuridine
cD1	Cyclin D1
Cdk	Cyclin-dependent kinase
Chk	Checkpoint kinase
CKI	Cyclin-dependent kinase inhibitor
CMV	Cytomegalovirus
DDR	DNA damage response
DIV	Days *in vitro*
DNA	Deoxyribonucleic acid
DSB	Double strand break
E	Embryonic day
EdU	5-ethynyl-2'-deoxyuridine
EGF	Epidermal growth factor
FACS	Fluorescence-activated cell sorting
FGF	Fibroblast growth factor
G1	Gap1 phase of the cell cycle
G2	Gap2 phase of the cell cycle
GADD45	Growth arrest and DNA-damage-inducible 45
GFP	Green fluorescent protein
GGF	Glial growth factor
IHC	Inner hair cell
IR	Ionizing radiation
Mdm	Murine double minute
M-phase	mitosis
MRN	MRE-11-Rad50-NBS1 complex
mRNA	Messenger ribonucleic acid
OHC	Outer hair cell
P	Postnatal day
PAR chain	poly(ADP-ribose) chain
PARP	poly(ADP-ribose) polymerase
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PH3	Phospho histone 3
Pou3f4	Human brain-4 ortholog POU3F4 (POU domain, class 3, transcription factor 4)

Prox1	Prospero-related homeobox 1
q-PCR	Quantitative polymerase chain reaction
Rb	Retinoblastoma
siRNA	Short interfering ribonucleic acid
Sox2	Sex determining region Y-box 2
Sox9	Sex determining region Y-box 9
S-phase	DNA synthesis -phase
SSB	Single strand break
UV	Ultraviolet
Wnt	Vertebrate homologue of *Drosophila* wingless gene
ZNPC	Zone of non-proliferating cells

In the text, gene names are written in *italics* and protein names in roman.

Abstract

Sensory hair cells and supporting cells of the mammalian inner ear are non-dividing cells, in contrast to supporting cells of non-mammalian species that have a natural capacity for cell cycle re-entry and production of new hair cells following traumas. The mechanisms regulating the maintenance of the postmitotic state of hair cells and supporting cells are not fully understood. In this thesis, the focus has been on understanding the involvement of the core cell cycle machinery in the regulation of the postmitotic state in the inner ear sensory epithelia. The knowledge of the mechanisms and molecules that regulate the postmitotic state of hair cells and supporting cells is of great importance when designing approaches to induce regeneration in the mammalian inner ear.

Cyclin dependent kinase inhibitors (CKIs) are negative cell cycle regulators that inhibit the progression of cell cycle. Earlier studies have shown that two CKIs, $p19^{Ink4d}$ and $p21^{Cip1}$, are expressed in postnatal hair cells, but the inactivation of either factor alone alters only mildly the postmitotic state of hair cell ($p19^{Ink4d}$ -/-) or does not have any effect ($p21^{Cip1}$ -/-). As it has been shown in several other cell types, like neurons, that CKIs often act redundantly to control the postmitotic state of quiescent cell, we hypothesised that there might be redundancy between $p19^{Ink4d}$ and $p21^{Cip1}$ in the inner ear as well. To study the possible co-operative role of $p19^{Ink4d}$ and $p21^{Cip1}$ in the regulation of the postmitotic state of hair cells, we generated a double knock-out (dko) mouse line, where these two CKIs are simultaneously inactivated (article I). The codeletion of $p19^{Ink4d}$ and $p21^{Cip1}$ led to robust cell cycle re-entry of auditory hair cells during a restricted period in early postnatal life. Part of the hair cells that had re-entered the cell cycle also progressed to mitosis and gave rise to new, ectopic hair cells. However, hair cell loss followed soon after cell cycle re-entry. Hair cell loss was a result of the induction of DNA damage following abnormal proliferation and activation of the DNA damage response pathway which led to the activation of tumour suppressor p53 and finally to apoptosis. These results show that $p19^{Ink4d}$ and $p21^{Cip1}$ act in co-operation to maintain the postmitotic state of auditory hair cells.

In addition to CKIs, we wanted to study the involvement of other cell cycle regulatory proteins in the regulation of the postmitotic state of inner ear sensory epithelial cells (article II). We found that cyclin D1 (cD1) is expressed transiently in postnatal cochlear hair cells. This transient expression pattern corresponded temporarily and spatially to the abnormal proliferation pattern seen in the dko mice. This suggested that both CKIs and cD1 are involved in the control of the postmitotic state of hair cells. We tested this hypothesis by ectopically expressing cD1 in vestibular hair cells where it is not normally expressed. Only vestibular hair cells from dko, but not from wild type mice re-entered the cell cycle in response to ectopic cD1 expression, confirming our hypothesis. Our

results showed that the control of the postmitotic state is a complex interplay between negative and positive cell cycle regulators.

The studies by us and others have shown that auditory hair cells are extremely sensitive to forced cell cycle re-entry and they rapidly die following cell cycle activation. In contrast, non-mammalian supporting cells are naturally capable of re-entering the cell cycle and producing replacement cells after trauma, and even neonatal mammalian vestibular supporting cells show proliferative potential in response to mitogens. We found that cD1 is strongly expressed in the neonatal vestibular supporting cells, but the expression declines rapidly thereafter. Interestingly, cD1 downregulation in vestibular supporting cells during the early postnatal life closely parallels the stage when these cells show a steep decline in mitogen responsiveness. This correlation suggested that the inability of mature supporting cells to re-enter the cell cycle could be caused by the absence of cD1 in these cells. We wanted to study if ectopically expressed cD1 could override this barrier (article III). We found that ectopically expressed cD1 triggered robust cell cycle re-entry of adult vestibular supporting cells. However, only a small fraction of supporting cells progressed into mitosis. The majority of cell cycle reactivated supporting cells showed DNA damage and arrested at the G2/M boundary.

Our studies show that the maintenance of the postmitotic state in mammalian hair cells and supporting cells is a complex process involving several cell cycle regulators. When hair cells are forced to re-enter the cell cycle, they react like several other quiescent cell types and rapidly undergo cell death. Supporting cells, especially vestibular supporting cells, have long been thought to be more plastic than hair cells. Our studies confirm that, as ectopic cD1 expression alone is sufficient to force supporting cells unlike hair cells to re-enter the cell cycle. However, despite the plasticity of supporting cells their proliferative potential is also restricted. Thus, it is clear that there are several challenges to overcome before regeneration in the mammalian inner ear is realized.

1. INTRODUCTION

Hearing loss is the most common sensory disorder in humans. Significant hearing loss occurs in about 15% of the world´s population. The great majority of human sensorineural hearing loss is caused by abnormalities in the sensory hair cells of the organ of Corti in the cochlea. Hearing loss can be due to genetic or environmental factors, such as noise, infections and ototoxic drugs. Mammalian hair cells do not regenerate during postnatal life and thus, the loss of each sensory cell is irreversible and cumulative. Hair cell can be lost also from the vestibular (balance) sensory epithelia of the inner ear leading to permanent balance dysfunction. In most of the cases, hearing loss or balance dysfunction cannot be medically or surgically corrected.

In lower vertebrates, such as birds and fishes, lost hair cells are replaced by new ones (Corwin and Cotanche 1988, Ryals and Rubel 1988). Regeneration in lower vertebrates occurs through division and transdifferentiation of non-sensory supporting cells (Corwin and Cotanche 1988, Ryals and Rubel 1988). Why mammals have lost the ability to regenerate lost hair cells, is not clear. One way to revive the regeneration in the mammalian inner ear is to induce the production of new hair cells: either by inducing the production of new hair cells from remaining hair cells or from supporting cells. In order to induce regeneration in the inner ear, it is crucial to first understand the special features of hair cells and supporting cells. Differentiated hair cells and supporting cells are postmitotic (Ruben 1967). Thus, understanding how the postmitotic state is regulated and how these highly specialized cells react when they are forced to re-enter the cell cycle, is an important step on the way to induce regeneration.

2. REVIEW OF THE LITERATURE

2.1 Structure of the mammalian inner ear

A mature mammalian inner ear is a complex structure that consists of two functionally different compartments: the vestibular system and the cochlea (Fig. 1A). The vestibular compartment houses five different sensory organs: three ampullae, located at the base of each semicircular canal, a utricle and a saccule. The vestibular sensory epithelia mediate balance function as they detect linear and angular head movements and head tilt. The sensory epithelium responsible for hearing function, the organ of Corti, resides in the cochlea. The inner ear sensory epithelia consist of two different cell types: hair cells and supporting cells. Both of these cell types are essential for inner ear function. Hair cells and supporting cells develop from common precursors, which exit the cell cycle during embryonic development. The common origin of hair and supporting cells was originally proven by retroviral tracing studies in the chick auditory organ (Fekete et al. 1998). Although no direct hair cell-lineage study is available in the mammalian inner ear, it is generally believed that these cell types arise from a common origin in mammals, as well. After reaching the postmitotic state precursors start to differentiate either towards the hair cells or supporting cells.

The cytoarchitecture of the auditory sensory epithelium, the organ of Corti, is much more complex than the cellular arrangement in the vestibular sensory epithelia (Raphael and Altschuler 2003). The organ of Corti consists of two types of hair cells, inner and outer hair cells, and four types of supporting cells: Claudius', Hensen's, Deiter's and pillar cells (Fig. 1B). Hair cells are mechanosensory cells that have a stereociliary bundle on their apical surface. The displacement of stereocilia, caused by sound waves, triggers a cascade of events that leads to voltage change across the hair cell membrane and finally to the release of neurotransmitters at the basal end of the cell. The neurotransmitters released by hair cells stimulate the neurons of the vestibulocochlear nerve (8th cranial nerve). The transduction of auditory stimuli into nerve impulses is accomplished primarily by the population of inner hair cells, which receive over 95 % of the afferent nerve fibers (Corwin and Warchol 1991). Rather than working as transducers, outer hair cells appear to function primarily as signal amplifiers. Efferent nerve fibers synapse to outer hair cells and regulate the contractive motion of these cells. The contraction of outer hair cells amplifies the motion of the sensory epithelium and creates greater distortion in the stereociliary bundles of the inner hair cells (Corwin and Warchol 1991). All four types of cochlear supporting cells have their specific features. Pillar cells are long columnar cells that are located between the inner and outer hair cells. In the mature organ of Corti, a tunnel structure, called the tunnel of Corti, is formed between the long cell bodies of pillar cells. Three Deiter's cells are located beneath the outer hair cells above the basement membrane. Hensen's and Claudius' cells are located lateral to the hair cells. The precise cellular arrangement of the cochlear hair cells and supporting cells is critical for the function of the organ of Corti (Raphael and Altschuler 2003).

The vestibular sensory epithelia consist of two types of hair cells and one type of supporting cell. Unlike in the organ of Corti, vestibular supporting cells are not morphologically highly differentiated. Instead, they are arranged tightly next to each other just above the basement membrane.

Vestibular hair cells are located above the supporting cell layer (Fig. 1C) (Beisel et al. 2005). Type I and II hair cells can be distinguished by their morphology and neuronal innervation. Wersäll (1956) described two classes of mammalian vestibular hair cells, distinguished principally by the kind of postsynaptic contact made by vestibular afferent fibers. On type II hair cells, the primary afferents form small rounded ("bouton") contacts similar to the afferent contacts formed on all auditory hair cells. Type I hair cells, in contrast, bear a cup-shaped afferent ending, or "calyx" (Wersäll 1956). The principles of hair cell function are largely similar between the hair cells in the vestibular sensory epithelia and the hair cells in the organ of Corti.

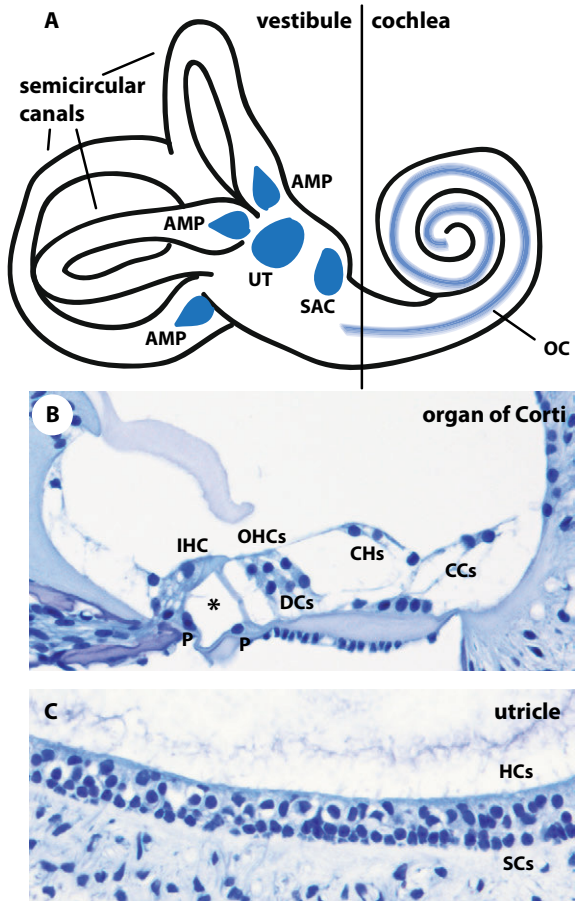

Figure 1. Structure of the inner ear labyrinth and sensory epithelia. (A) Schematic picture of the inner ear labyrinth. Two functionally different compartments, vestibule and cochlea, can be distinguished. At the base of each semicircular canal resides one ampulla (AMP). The sensory epithelia in the ampullae are called ampullary cristae. Utricle (UT) and saccule (SAC) are located medially to ampullae and the sensory epithelia are called maculae. The auditory sensory epithelium, organ of Corti (OC), resides in the cochlea. (B) Hematoxylin stained transverse section of the mature organ of Corti. Different cell types are indicated: IHC=inner hair cell, OHCs= outer hair cells, P=pillar cell, DCs= Deiter's cells, CHs= Hensen's cells and CC=Claudius' cells. Asterisk (*) indicates the tunnel of Corti, formed between the long cell bodies of pillar cells. (C) Hematoxylin stained transverse section of the utricle. Supporting cells (SCs) are arranged next to each other at the base of the epithelium and hair cells (HCs) are located above the SC layer.

2.2 Cell cycle regulation

The cell cycle is a highly conserved and ordered set of events, culminating in cell growth and division. The cell cycle is tightly controlled by many regulatory mechanisms that either permit or restrain its progression. The cell cycle consists of four phases; G1, S (DNA synthesis), G2, and M (mitosis) (Fig. 2). The key families that comprise the basic regulatory machinery responsible for controlling cell cycle progression are cyclins, cyclin dependent kinases (Cdks), Cdk inhibitors (CKIs), the tumour suppressor protein p53, and the retinoblastoma protein family (Rb, p107, and p130).

Cyclins are the regulatory subunits of the holoenzyme cyclin dependent kinase complexes. Many hormones and growth factors influence cell growth through signal transduction pathways that modify the activity of cyclins. The Cdks are a family of Ser/Thr protein kinases that act by phosphorylating different substrates required for cell cycle progression (Morgan 1997). More than 20 Cdk proteins have been identified in mammalian cells, but not all Cdks are directly involved in cell cycle regulation. In mammals, there are four major classes of cyclins: A (including A1 and A2), B (including B1, B2 and B3), D (including D1, D2 and D3) and E (including E1 and E2) (Sherr 1993, Pines and Hunter 1994). According to the classical model of cell cycle regulation, each Cdk/cyclin complex harbours unique functions restricted to a particular phase of the cell cycle (Fig. 2). According to this model, in early G1 phase Cdk4 and/or Cdk6 are activated by D-type cyclins and initiate phosphorylation of the retinoblastoma protein family (Sherr and Roberts 1999, 2004). This in turn leads to the release of E2F transcription factors that activate the transcription of genes required for cell cycle progression, such as E- and A-type cyclins (Weinberg 1995, Dyson 1998). In the late G1 phase, cyclin E activates Cdk2. Cdk2/cyclin E complex completes the phosphorylation on Rb leading to further activation of E2F mediated transcription and passage through the restriction point at the boundary of G1/S phase (Sherr and Roberts 1999, 2004). In the S phase, Cdk2 complexes with cyclin A and phosphorylates proteins required for DNA replication (Petersen et al. 1999, Coverley at al. 2000). During the G2/M transition, Cdk1/cyclin A activity is required (Furuno et al. 1999) and finally, Cdk1 acts together with cyclin B to complete mitosis (Riabolow et al. 1989). During the last decade, this classical model has been challenged due to the uncovering of several unexpected compensatory mechanisms among cyclins and Cdks (Satyanarayana and Kaldis 2009). Under normal circumstances, the functions of Cdks are limited to a particular phase of the cell cycle; however, in the absence of one or more Cdks, the remaining Cdks will take over the functions of lost Cdks and control the cell cycle progression faithfully. This same compensatory action also applies to different cyclins (Satyanarayana and Kaldis 2009). One of the most remarkable examples of compensation among Cdks is the observation that in the absence of Cdk2, 3, 4, and 6, Cdk1 can alone drive the cell cycle by complexing with all the phase-specific cyclins (Santamaria et al. 2007). Mouse embryos lacking all interphase Cdks (Cdk2, Cdk3, Cdk4 and Cdk6) undergo organogenesis and develop to midgestation when they die due to severe haematopoietic defects (Santamaria et al. 2007). Although there is strong evidence on the compensation among Cdks and cyclins, some animal models deficient for only one Cdk or cyclin show cell type or tissue specific defects or abnormalities at certain stages of development. It has been demonstrated that for example the Cdk2 deficiency impairs the proliferation of neural progenitor cells of the subventricular zone in the adult animals, although they do not require Cdk2 prenatally (Jablonska et al. 2007). This shows that *in vivo*, the compensation by another Cdk or cyclin is not

always sufficient to drive normal cell cycle progression and the need for different cyclins and Cdks can be highly tissue specific or specific to the certain developmental stage.

The activities and functions of Cdk/cyclin complexes are regulated by two families of Cdk inhibitors (CKIs): the INK4 family and Cip/Kip family (Fig. 2). The INK4 family consists of four members: $p16^{Ink4a}$ (Serrano et al., 1993), $p15^{Ink4b}$ (Hannon and Beach, 1994), $p18^{Ink4c}$ (Guan et al., 1994, Hirai et al., 1995) and $p19^{Ink4d}$ (Chan et al., 1995, Hirai et al., 1995). The Cip/Kip family includes three members: $p21^{Cip1}$ (Gu et al. 1993, Harper et al. 1993, El-Deiry et al. 1993, Xiong et al. 1993, Dulić et al.1994) $p27^{Kip1}$ (Polyak et al. 1994a,b, Toyoshima and Hunter 1994) and $p57^{Kip2}$ (Lee et al. 1995, Matsuoka et al. 1995). INK4 family members specifically bind to Cdk4 and Cdk6, and prevent D-type cyclin activity. Cip/Kip family members inhibit Cdk2/cyclin E, Cdk2/cyclin A, Cdk1/cyclin A, and Cdk1/cyclin B activities. It has been shown in several tissues that the precise regulation of CKI expression is critical for appropriate development, and the breakdown of this regulatory mechanism is deeply involved in oncogenesis. The function of CKIs is discussed in more detail in paragraph 2.2.2.

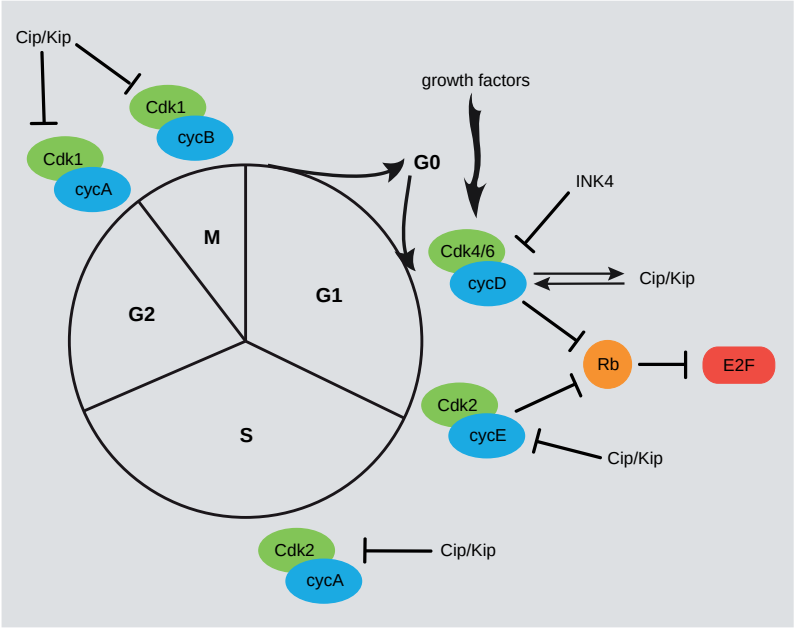

Figure 2. The classical model of cell cycle regulation. Under normal circumstances different cyclin dependent kinases (Cdks, green ovals) are paired with different regulatory subunits, cyclins (cyc, blue ovals), during different phases of the cell cycle (G1, S, G2 and M). The activity of Cdk-cyclin complexes is regulated by two families of cyclin dependent kinase inhibitors (CKIs): the INK4-family and Cip/Kip-family. The INK4 family inhibits only Cdk4/6-cycD complexes, whereas Cip/Kip family members inhibit the other Cdk/cyc pairs. For Cdk4/6-cycD complexes, the Cip/Kip proteins are essential for their assembly. This interaction between Cdk4/6-cycD and Cip/Kip proteins is also important to lower the levels of unbound Cip/Kip proteins and thus, it facilitates the activation of Cdk2/cycE complexes, which are targets of Cip/Kip mediated inhibition (see chapter 2.2.1 for details). Extracellular signals, like growth factors, regulate the cell cycle progression through the induction of cyclin D (cycD). The critical step in cell cycle progression is the phosphorylation (inactivation) of the Rb protein (orange) by Cdk4/6-cycD and later by Cdk2-cycE complexes. Once pRb is phosphorylated, E2F (red) is released, enabling the transcription of genes required for cell cycle progression.

The tumour suppressor protein p53 is also a critical cell cycle regulator. One the roles of p53 is to respond to various forms of DNA damage by either arresting the cell cycle to allow adequate DNA repair or kill the cell (Kastan et al. 1991, Kuerbitz et al. 1992). Thus, p53 is involved in the cell cycle checkpoint pathways that monitor DNA damage and replication, and thereby regulate progression through the cell cycle. In the eukaryotic cell cycle, there are three main cell cycle checkpoints: G1/S checkpoint, intra-S-phase checkpoint, and G2/M checkpoint (Elledge 1996). Checkpoint pathways and the role of p53 are discussed in more detail in paragraph 2.2.3.

2.2.1 D-type cyclins

D-type cyclins have an important role in the cell cycle, where they act as growth factor sensors. Cyclin D transcription, assembly, nuclear transport and turnover are all mitogen-dependent steps (Sherr 1993). The most recognized function of cyclin D-dependent kinases is phosphorylation of pRb (Matsushime et al. 1994). A second, noncatalytic function of Cdk4/cyclin D complexes is sequestration of CKIs of the Cip/Kip family, including $p27^{Kip1}$ and $p21^{Cip1}$ (Xiong et al. 1993, Polyak et al. 1994a, Toyoshima and Hunter 1994). This interaction between Cdk4/cyclin D complex and $p27^{Kip1}$ or $p21^{Cip1}$ is an important regulatory step in G1/S transition. Sequestration of unbound Cip/Kip proteins lowers the inhibitory threshold and facilitates the activation of Cdk2/cyclin E complexes, which are targets of Cip/Kip mediated inhibition (see Fig. 2). The interaction of Cdk/cyclin D with CKIs is not only important to lower the levels of free CKI molecules, but this interaction also facilitates the activation of Cdk/cyclin D complexes (LaBaer et al. 1997). It has been shown that the assembly of Cdk4/cyclin D1 complexes is impaired in primary mouse embryonic fibroblasts (MEFs) derived from the animals lacking either *p21 Cip1* gene, p27 *Kip1* gene, or both (Cheng et al. 1999). In addition to facilitating the assembly of Cdk/cyclin D complexes, Cip/Kip proteins also promote their activation by inducing the nuclear import and stabilization of D-type cyclins (LaBaer et al. 1997, Cheng et al. 1999).

The cyclin D family includes three members: D1, D2 and D3. D-type cyclins are expressed in a variety of cell types and tissues with cyclin D1 being the most ubiquitously expressed (Waclaw and Chatot 2004). Mice lacking individual D-type cyclins are viable, develop normally and do not show any severe impairment in cell proliferation, but display several cell type specific abnormalities (Kozar et al. 2004). *Cyclin D1 -/-* mice have reduced body size, display hypoplastic retinas and neurological abnormalities. In addition, the mammary glands of females fail to undergo lobuloalveolar development during pregnancy (Sicinski et al. 1995, Fantl et al. 1995). As the lack of an individual D-type cyclin does not have a dramatic effect on cell proliferation and survival in the majority of cell types, it appears that other D-type cyclins are able to substitute the missing one in most cases. However, the minor cell type specific defects indicate that other cyclin D family members are not able to fully compensate the loss of other D-type cyclins in certain cell types. This is most probably due to the lack of expression or differences in the timing of expression of each of the D-type cyclins in a particular cell type. In support to this view, it has been shown that when cyclin D2 is expressed from the cyclin D1 locus, it can fully rescue the loss of cyclin D1 (Carthon et al. 2005). When all three D-type cyclins are deleted, cells can still divide and progress through different phases of the cell cycle and the mice can survive until midgestation (Kozar et al. 2004).

This shows that wide range of intra- and interfamily compensatory mechanisms among cyclins clearly exist, as they do also among different Cdks.

In many cell types, induction of the expression of cyclin D1 by extracellular signals appears to be a key intracellular event that regulates passage through the G1 phase (Sherr and Roberts 1999). Subsequent phosphorylation of pRb represents a step when the cell becomes committed to a new round of cell division. Transgenic mice with targeted overexpression of cyclin D1 in different cell types demonstrate enhanced proliferation during development (Wang et al. 1994, Robles et al. 1996, Nakagawa et al. 1997, Rodrigues-Puebla et al. 1999). Interestingly, Nelsen et al. (2001) showed that in the adult mice cyclin D1 is also sufficient to trigger proliferation of quiescent, differentiated cells. In this study hepatocytes were used as a model. In normal adult liver, hepatocytes are highly differentiated and rarely undergo division, but they retain a remarkable ability to proliferate in response to acute or chronic injury (Fausto 2000). However, it still remains unclear, whether cyclin D1 is sufficient to induce proliferation in other quiescent cell types, especially in those that do not have proliferative potential intrinsically.

2.2.2 CKIs and the regulation of postmitotic state

In many cell types and tissues, CKIs are essential for normal development and maintenance of a differentiated state. In several cell types, like neurons and myocytes, the cell cycle exit of progenitor cells precedes cellular differentiation and maturation (Buttitta and Edgar 2007). In addition to the cell cycle exit of progenitors, CKIs are also involved in the maintenance of the postmitotic state of differentiated cells, which is an equally important process for the correct function of the cell or whole tissue.

The Cip/Kip family of CKIs is phylogenetically well conserved. Despite several structural and biochemical similarities among the Cip/Kip members, the phenotypes of knockout mice of each Cip/Kip member are very different, which suggest that the Cip/Kip CKIs have a variety of physiological functions that other family members cannot always fully compensate. The family members $p27^{Kip1}$ and $p57^{Kip2}$ share several similarities in their structure and function; however, their spatial and temporal expression patterns in mice are substantially different (Nagahama et al. 2001). Whereas the expression of $p57^{Kip2}$ is observed in specific cell types and restricted to embryogenesis, the expression of $p27^{Kip1}$ is widely distributed in many tissues and maintained in adult animals. The phenotypes of mice lacking *p27^{Kip1}* or *p57^{Kip2}* also differ markedly. Whereas *p27^{Kip1}* knockout mice manifest an increased body size, multiple organ hyperplasia, and increased tumour development (Kiyokawa et al. 1996, Fero et al. 1996, Nakayama et al. 1996), all of which are consistent with the cellular function of $p27^{Kip1}$ as a CKI, *p57^{Kip2}* knockout mice show severe developmental defects in several tissues including the palate, lens, kidney, intestine, bone and abdominal wall, with most of the animals dying just after birth (Zhang et al. 1997, Yan et al. 1997, Takahashi et al. 2000). Unexpectedly, an increased frequency of apoptosis, rather than overproliferation as seen in *p27^{Kip1}* knockout mice, is apparent in the affected tissues of the *p57^{Kip2}* knockout animals (Zhang et al. 1997, Yan et al. 1997). These findings indicate that $p57^{Kip2}$ might also have functions that are not directly related to its action as a CKI; rather, $p57^{Kip2}$ could also have roles in cellular differentiation and survival processes. Quite recently a knock-in mouse model in which the *p57^{Kip2}* gene is replaced by the *p27^{Kip1}* gene was generated (Susaki et al. 2009). The knock-in of

the *p27 Kip1* gene in place of the *p57*^{Kip2} gene greatly ameliorated the mortality and developmental defects of *p57Kip2* knockout mice. Some defects of *p57Kip2* knockout mice, including those of the renal papilla, placenta and abdominal wall muscle, remained in the *p27Kip1 →p57Kip2* knock-in mice, indicating that p57^{Kip2} has specific functions in certain tissues (Susaki et al. 2009). The p57^{Kip2} protein contains domains (proline domain and acidic domain) that do not exist in p27^{Kip1} or p21^{Cip1}, and these domains might be related to the unique functions of p57^{Kip2} (Matsuoka et al. 1995, Lee et al. 1995).

Compared to other Cip/Kip family members, p21^{Cip1} is structurally and functionally different (Goubin et al. 1995). Unlike other Cip/Kip family members, p21^{Cip1} functions as a dual specificity inhibitor, as it does not only bind to Cdk/cyclin complexes, but it also associates with the DNA replication factor PCNA (proliferative cell nuclear antigen) (Flores-Rozas el al. 1994, Warbrick et al. 1995). In addition to these functions, p21^{Cip1} is a well known target of p53 (Macleod et al. 1995). Myogenesis is a classic example of a developmental circuit where cell cycle exit, which requires CKIs, and terminal differentiation are connected. During myogenesis p21^{Cip1} has been shown to be activated by MyoD, a bHLH (basic helix-loop-helix) transcription factor that is sufficient to drive muscle cell differentiation (Parker et al. 1995, Halevy et al. 1995). Before terminal differentiation is initiated, the activity of Cdk/cyclin complexes maintains differentiation factors, like MyoD, in an inactive state. Evidence for this comes from the finding that forced expression of cyclin D1 in myoblasts under differentiation-inducing conditions can maintain MyoD in its non-functional form (Rao et al. 1994). Once a differentiation initiation signal is received, MyoD is activated and it induces the expression of p21^{Cip1} and other genes involved in the initiation of the differentiation program. Despite the fact that p21^{Cip1} is induced during myoblast differentiation, *p21Cip1* null mice develop normally and do not show any muscle specific defects (Deng et al. 1995). In contrast to *p21Cip1* single null mice, mice harbouring simultaneous deletion of *p21Cip1* and *p57*^{Kip2} show severe impairment in myoblast fusion and abnormal proliferation and apoptosis of myoblasts (Zhang et al. 1999). This indicates again that in several systems there is redundancy between different CKIs.

The four proteins of the INK4 family share a similar structure dominated by several ankyrin repeats (Jeffrey et al. 2000). Although they appear to be structurally redundant and equally potent as inhibitors, the INK4 family members are differentially expressed during mouse development (Zindy et al. 1997). The diversity in the pattern of expression of INK4 genes suggests that this family of cell cycle inhibitors harbours cell lineage-specific or tissue-specific functions similarly to the Cip/Kip family. The INK4 proteins are commonly lost or inactivated by mutations in diverse types of cancer and they represent established or candidate tumour suppressors. p18^{Ink4c} and p19^{Ink4d} are the most important INK4 family members related to embryonic and early postnatal development in certain tissues (Zindy et al. 1997). Despite the fact that p19^{Ink4d} shows widespread expression during development, *p19Ink4d* null mice develop normally and do not develop tumours. One of the defects caused by the depletion of *p19Ink4d* is testicular atrophy in male mice (Zindy et al. 2000). Although *p19Ink4d* loss partially compromises germ cell development in male mice, their sperm counts remain at sufficient levels to maintain fertility. When both *p18Ink4c* and *p19Ink4d* genes are disrupted, testicular function is more severely impaired resulting in increased levels of germ cell apoptosis, fewer spermatozoa, and infertility (Zindy et al. 2001).

As many examples have shown, CKIs within the same family often have redundant functions. Redundancy can be also observed between CKIs from different families. $p19^{Ink4d}$ and $p27^{Kip1}$ show redundant functions in several neuronal populations (Zindy et al. 1999) and in certain retinal cell populations (Cunningham et al. 2002). *$p19^{Ink4d}$ /$p27^{Kip1}$* deficient mice develop normally until the second postnatal week when several neurological abnormalities (bradykinesia and seizures) appear. BrdU pulse labelling experiments show proliferation of differentiated neurons in several brain areas, like hippocampus, cerebral cortex, hypothalamus and pons. Abnormal proliferation is rapidly followed by apoptosis, and ultimately these mutant mice die around P18 (Zindy et al. 1999). The retinal phenotype of *$p19^{Ink4d}$ /$p27^{Kip1}$* deficient mice closely resembles the neuronal phenotype: several differentiated retinal cell types enter the cell cycle and following cell cycle re-entry undergo apoptosis (Cunningham et al. 2002). It seems that in certain cell populations, such as neurons, abnormal cell cycle activity is coupled to cell death. In the study by Cunningham et al. (2002) it was shown that the inactivation of *p53* partially rescues the cell death phenotype in *$p19^{Ink4d}$ /$p27^{Kip1}$* deficient retinas indicating the involvement of p53, but apparently also other factors, in apoptosis related to abnormal proliferation.

2.2.3 DNA damage response (DDR) and p53

Damage to the genetic material is a severe threat to every cell. DNA damage can be caused by environmental factors, such as ionizing radiation (IR), ultraviolet (UV) light, chemical agents, or errors during DNA replication. Several types of DNA damage exist, i.e. base modifications, double strand breaks (DSBs) and single strand breaks (SSBs). Eukaryotes have evolved a complex signal transduction pathway called DNA damage response (DDR) that has the ability to sense DNA damage and replication stress, and to respond to it in various ways. The final response can be cell cycle arrest and DNA repair or cell death, depending on the severity of DNA damage and the cell type experiencing the damage (Zhivotovsky and Kroemer 2004).

DNA lesions are first recognised by sensor proteins (Fig. 3). There are several proteins that can act as sensors, but their function is not fully understood. The Mre11-Rad50-Nbs1 (MRN) complex forms at DNA double strand break sites (DSBs) (D'Amours and Jackson 2002). Following the formations of single strand breaks (SSBs), DNA damage sensors Rad9, Hus1, and Rad1 form a ring structure (the 9–1–1 complex) that binds to the DNA damage site (Roos-Mattjus et al. 2002). In addition, PARP family members (especially PARP1 and PARP2, poly(ADP-ribose) polymerase 1 and 2) are important molecular sensors of DNA single strand breaks (SSBs) and double strand breaks (DSBs) (Smulson et al. 2000). PARP proteins catalyze the synthesis of poly(ADP-ribose) (PAR) chains at the damage sites and recruit other DDR factors. Thus, the important function of sensor proteins is to recognize damaged DNA and to form a scaffold for downstream checkpoint proteins.

Following the recognition of DNA lesions by sensor proteins, ATM (ataxia telangiectasia mutated) and ATR (ataxia telangiectasia and rad3-related) kinases are recruited to the damage site. ATM and ATR act by recruiting other DDR factors to sites of DNA damage (Kastan and Bartek 2004). ATM and other PI3-like kinases also phosphorylate a member of the histone H2A family, H2AX (γH2AX) (Fig. 3). Phosphorylation of H2AX occurs rapidly after the induction of DNA damage, but the exact reason for this phenomenon is not yet clarified, although a role in

DNA damage repair and chromosomal stability is evident (Rogakou et al., 1998). The detection of these γH2AX foci is a common way to visualize DSBs in cells. Effector proteins of the DDR are either directly phosphorylated by ATM/ATR or by the checkpoint kinases Chk1 (ATR target) and Chk2 (ATM target), as well as other kinases such as CK2, p38, and MK2 (Harper and Elledge, 2007). There are several effector proteins, depending on the response pathway. These effectors are either directly or indirectly involved in cell cycle regulation and their activation arrests the cell cycle or induces apoptosis.

While the DDR is primarily mediated through fast posttranslational modifications, such as phosphorylation, slower transcriptionally mediated regulatory steps are also involved. One of the most important targets regulated by ATM and Chk2 in response to DSBs is p53 (Kastan et al. 1991, Kastan and Bartek 2004) (Fig. 3). These kinases can directly activate p53 by phosphorylation or indirectly by mediating the destruction of two important inhibitors of p53, Mdm2, and Mdm4 (murine double minute 2 and 4). Mdm2 acts as an ubiquitin ligase and mediates p53 destruction. In contrast, Mdm4 inhibits p53 activation by limiting its access to essential transcriptional co-activators and to the basal transcription machinery (Toledo et al. 2006, Francoz et al. 2006). p53 induces cell cycle arrest, apoptosis, or senescence by transcriptionally regulating several targets such as $p21^{Cip1}$, GADD45 (growth arrest and DNA-damage-inducible 45), 14-3-3σ and the proapoptotic proteins BAX and PUMA (Riley et al. 2008). In addition, p53 has transcription-independent functions in mitochondria that can induce apoptosis (Chipuk et al. 2004) (Fig. 3).

While a number of studies have investigated the responses of proliferating cells to genotoxic stress, the DNA damage response in terminally differentiated cells, like neurons, is poorly understood. Increasing evidence indicates that neurodegeneration involves the activation of cell cycle machinery in postmitotic neurons (Copani et al. 2001). A study by Kruman et al. (2004) showed that cell cycle activation is essential for DNA damage-induced neuronal apoptosis. Furthermore, the activation of the cell cycle machinery plays a role in the repair of damaged DNA in terminally differentiated neurons and thus contributes to neuronal survival (Schwartz et al. 2007). Terminally differentiated cells, including neurons, have generally very poor DNA repair capability, and probably for that reason they are more prone to DNA damage-initiated apoptosis rather than DNA repair.

DNA damage induced hair cell death and the involvement of p53 in this process has been demonstrated in *in vitro* studies where auditory and vestibular hair cells were exposed to cisplatin (Zhang et al. 2003). Cisplatin is an anti-cancer drug that induces the formation of DSBs. Inner ear hair cells are particularly sensitive to this drug. In the study by Zhang et al. (2003), it was shown that upon cisplatin treatment, p53 is activated in auditory and vestibular hair cells. When a p53 inhibitor (pifithrin-α) was applied together with cisplatin, hair cells were protected from apoptosis. Whether DNA damage induced hair cell death and activation of p53 is preceded by cell cycle activation, as has been reported in neuronal cells (Kruman et al. 2004), remains to be studied.

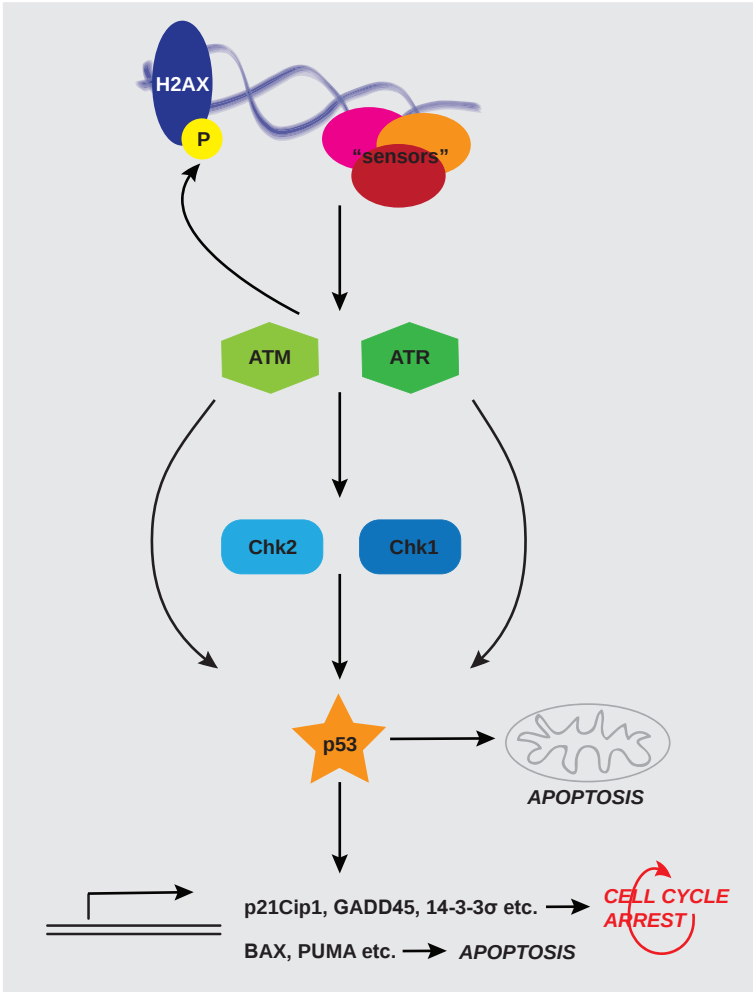

Figure 3. Main components of the DDR pathway leading to activation of p53. DNA lesions are recognised by sensor proteins ("sensors"). There are several different sensors depending on the type of DNA damage (see text for details). After the recognition of DNA damage, ATM and ATR kinases (green) are recruited to the site of damage and activated. Histone H2AX is phosphorylated by ATM/ATR kinases. p53 (star) is activated by ATM/ATR kinases or by Chk kinases (blue). These kinases can directly activate p53 by phosphorylation or indirectly by inactivating proteins that inhibit p53 (Mdm2 and 4, not indicated in the picture). p53 regulates transcriptionally several target genes which can either induce cell cycle arrest ($p21^{Cip1}$, GADD45, 14-3-3) or apoptosis (BAX, PUMA). In addition, p53 can transcription-independently affect the function of mitochondria and thus activate apoptosis.

2.3 Cell cycle regulation in the inner ear

Inner ear hair cells and supporting cells are postmitotic. There are two important steps in the life cycle of quiescent cells. The first step is the initial cell cycle exit of progenitor cells, which is usually followed by cellular differentiation. The second step is the lifelong process of maintaining the postmitotic state, which is essential for the survival and function of these cells. In the next two paragraphs it will be discussed how these two important steps are taken and regulated in the inner ear sensory epithelia.

2.3.1 Cell cycle exit of inner ear sensory epithelial progenitors

Inner ear hair cells and supporting cells develop from common precursors. After the precursors have exited the cell cycle, differentiation into hair cells and supporting cells is initiated. The cell cycle exit is regulated by $p27^{Kip1}$, a member of the Cip/Kip family of cyclin-dependent kinase inhibitors (CKIs). In the organ of Corti, the expression of $p27^{Kip1}$ starts at embryonic day 12.5 (E12.5) from the most apical end of the cochlear duct (Lee et al. 2006). The wave of $p27^{Kip1}$ expression reaches the basal end by E14.5. By using BrdU incorporation studies, it has been shown that the wave of $p27^{Kip1}$ expression parallels or slightly precedes the wave of cell cycle exit (Lee et al. 2006). It is not known which upstream factors regulate *$p27^{Kip1}$*, but it is likely that Sox2, Notch1 and Jagged1 have a role since $p27^{Kip1}$ expression is lost or reduced in *Jag1* knockout and *Sox2* mutant mice (Kiernan et al. 2005a, 2006, Brooker et al. 2006, Dabdoub et al. 2008). Cells that express $p27^{Kip1}$ and exit the cell cycle form the zone of non-proliferating cells (ZNPC), which will later give rise to the organ of Corti. Thus, formation of the postmitotic, prosensory domain of the cochlear duct is largely completed at E14.5, the time of onset of the hair and supporting cell differentiation. Cellular differentiation in the organ of Corti requires the expression of Atoh1 (also called Math1), the mammalian homolog of the *Drosophila melanogaster* gene *atonal* (Bermingham et al.1999, Chen and Segil 1999). Atoh1 is a bHLH (basic helix-loop-helix) transcription factor. The induction of Atoh1 in hair cells occurs between E13.5 and E14.5 in the midbasal region of the cochlear duct from where it spreads towards the apical part reaching the apical tip around E17.5 (Bermingham et al. 1999, Chen and Segil 1999). As the pattern of cellular differentiation occurs in a direction opposing the pattern of cell cycle exit, the most apical sensory progenitors are maintained in an undifferentiated, but postmitotic state 4-5 days before differentiation occurs. In majority of the developing systems there is a very close relationship between cell cycle exit and onset of differentiation (Götz and Huttner, 2005, Nguyen et al. 2006). In fact, bHLH genes have been shown to concurrently antagonize cellular proliferation and induce cellular commitment and differentiation (Farah et al. 2000, Le et al. 2006, Battiste et al. 2007). Therefore, the presence of uncommitted, postmitotic prosensory cells represents the maintenance of a relatively rare cellular state. The factors that mediate this status and, more importantly, the biological basis for their existence are still unclear. Atoh1 is necessary for hair cell differentiation. In *Atoh1* null-mice hair cells are lacking from all sensory epithelia. In addition, *Atoh1* is sufficient to drive hair cell differentiation when it is misexpressed in nonsensory cells of the organ of Corti (Zheng and Gao 2000, Shou et al. 2003). The expression of Atoh1 in differentiating hair cells triggers an important signalling cascade which ultimately leads to the formation of a strictly arranged cellular mosaic where different types of hair cells and supporting cells are arranged in a specific pattern that is

essential for the function of this epithelium. Atoh1 induces the expression of Jagged2. Jagged2 in turn activates the Notch1 receptor in the neighbouring cells. Notch1 inhibits the expression of prosensory genes and this inhibition guides these cells to adopt a supporting cell fate (Lanford et al. 1999, Kiernan et al. 2005b). During differentiation, p27 ^{Kip1} expression is downregulated in hair cells, but it persists at high levels in differentiated supporting cells (Chen and Segil 1999, Löwenheim et al. 1999).

In contrast to their counterparts in the cochlea, the cells of the vestibular sensory organs withdraw from the cell cycle over a longer period of time, ranging from E14 to P3 (postnatal day 3) (Ruben 1967). p27^{Kip1} expression in vestibular sensory epithelia can be observed at E14 and this suggests that a similar sequence of developmental events involving p27^{Kip1} in cell cycle exit takes place in the vestibular system as it does in the organ of Corti (Chen and Segil 1999). Similarly to the organ of Corti, p27^{Kip1} expression persists also in the vestibular supporting cells of neonatal mice (Chen and Segil 1999). In contrast to cochlear hair cells, a small population of hair cells located in a scattered manner within the vestibular sensory epithelia show weak p27 ^{Kip1} expression neonatally (Chen and Segil 1999).

As can be expected, the inactivation of *p27 ^{Kip1}* has a dramatic effect on the development of the sensory epithelia (see Table 1). In mice homozygous for a *p27^{Kip1}* mutation (*p27*-/-), proliferation of progenitor cells in the auditory sensory epithelium continues after the normal cell cycle exit period (Chen and Segil 1999, Löwenheim et al. 1999). This abnormal proliferation leads to the formation of supernumerary hair cells and supporting cells. Proliferative activity can be seen in the supporting cell population, especially in Hensen's, Claudius' and pillar cells, even in adult mice, which is consistent with the fact that p27^{Kip1} expression persists in supporting cells. In the adult mouse(4 months) organ of Corti, the proliferative activity of supporting cells is significantly lower than during the early postnatal stages (Chen and Segil 1999, Löwenheim et al. 1999). On the basis of immunohistological stainings, the supernumerary hair cells are fully differentiated (express hair cell specific proteins, such as myosin VIIa) and appear to have the normal pattern of neuronal innervations. However, based on auditory brainstem response (ABR) measurements, these mutants are severely hearing impaired (Chen and Segil 1999). The supporting cells also show features of fully differentiated cells, like large microtubular bundles (Löwenheim et al. 1999). This indicates that even without p27^{Kip1}, hair cells and supporting cells are still able to stop dividing and differentiate. Thus, the hearing impairment in *p27^{Kip1}* mutant mice is probably related to the abnormal cellular arrangement in the organ of Corti caused by supernumerary hair cells and supporting cells. These results show that p27^{Kip1} has an important function in determining the size of progenitor population, but is probably not required for the terminal differentiation of hair or supporting cells. p27 ^{Kip1} also has an important role in controlling the postmitotic state of differentiated supporting cells. In general, *p27^{Kip1}* -/- mice display generalized hyperplasia in many tissues, as well as abnormalities in tissue organization. In spite of the increase in cell number, the function of most tissues is not grossly perturbed (Fero et al. 1996, Kiyokawa et al. 1996, Nakayama et al. 1996). This suggests that overlapping pathways that control the cell cycle during development most likely exist in several tissues. This also indicates a limited role for p27^{Kip1} in differentiation *in vivo* (Fero et al. 1996, Kiyokawa et al. 1996). *p27^{Kip1}* deficient mice show no obvious behavioural defects related to vestibular function, such as circling behaviour or balance problems, but the

effect of *p27^{Kip1}* inactivation on the cell cycle status of vestibular sensory epithelial cells has not been investigated in depth (Chen and Segil 1999).

2.3.2 Maintenance of the postmitotic state in inner ear sensory epithelia

p27^{Kip1} has a well established function during the initial cell cycle exit of inner ear sensory epithelial progenitors, as already discussed in the previous chapter. The maintenance of the postmitotic state of hair cells and supporting cells is also crucial for the function of the inner ear sensory epithelia. During the past few years, a number of studies have focused on understanding how the postmitotic state is maintained and which cell cycle regulators are involved in this process (see Table 1: Phenotypes of mutant mouse lines used in studies of the postmitotic state in the inner ear).

Several studies have investigated the role of pRb in the maintenance of the postmitotic state. The expression of pRb can be seen already in the sensory precursors at E12.5 (Mantela et al. 2005). *Rb (mgRb:Rb -/-)* inactivation delays the timing of terminal mitoses of precursors, leading to elevated precursor cell numbers. In addition, aberrant proliferation in differentiating hair cells of both vestibular and cochlear sensory epithelia was observed (see Table 1). As a result, the sensory epithelia were severely hyperplastic. Despite their abnormal cycling, *Rb* null hair cells showed hair cell specific gene expression (Atoh1, myosin VII and VI and calbindin) indicating that hair cell differentiation does not require pRb. When analyzed at birth, Rb null hair cells showed signs of apoptosis and multinucleation, indicating defects in cytokinesis (Mantela et al. 2005). As *mgRb:Rb -/-* mice die at birth, further investigation of hair cell fate was not possible with this model. Later Sage et al. (2006) developed conditional *Rb* knockout mice using a cre-lox strategy, in which cre was under the promoter of the transcription factor Pou4f3 leading to *Rb* inactivation in cochlear progenitor cells and vestibular hair cells. *Pou4f3-pRb-/-* mice (see Table 1) survived up to 6 months and showed total hair cell loss in the organ of Corti by 2 months, indicating that cell death is indeed the final fate of cochlear hair cells after *Rb* inactivation. According to this study, postnatal *Pou4f3-pRb-/-* auditory hair cells and supporting cells underwent cell division. In addition, hair cell maturation was disturbed and these maturational defects were suggested to cause the hair cell death. In the utricle, postnatal *Pou4f3-pRb-/-* hair cells continued to divide in 6-week-old mice. The differentiation of utricular hair cells was not affected and these hair cells showed long-term survival up to 6 months (Sage et al. 2006). Another study by Weber et al. (2008) showed that the response to *Rb* loss is dependent on the differentiation stage of the hair cells. When *Rb* was inactivated in postnatal hair cells *(*using *Atoh1-CreER*:*Rb*$^{lox/lox}$ mouse line, tamoxifen administrated at P0-P1, see Table 1), rapid cell cycle entry and rare mitotic figures were detected, but no supernumerary hair cells were produced. Rather cell cycle re-activation was immediately followed by apoptosis. To study the response of adult hair cells to *Rb* inactivation, another inducible *Rb* knockout mouse line was used by Huang et al. (2011). In this ER-Cre-pRb$^{flox/flox}$ mouse line (see Table 1), *Rb* can be acutely deleted postnatally. When *Rb* was inactivated in 3 month-old mice, no proliferation was observed either in hair cells or in supporting cells. This strongly suggests that additional mechanisms are involved in the regulation of the postmitotic state in the adult inner ear. The effect of *Rb* inactivation on postnatal supporting cells has been studied by using a mouse model where cre is under the Prox1 promoter (see Table 1). *Prox1* is a gene that is specifically expressed in two

subtypes of postmitotic cochlear supporting cells, pillar cells, and Deiter's cells (Yu et al. 2010). These Prox1-CreER mice were given tamoxifen at P0 and P1. When analyzed at P4, both pillar and Deiter's cells were seen to re-enter the cell cycle and also undergo mitosis. The mitotic activity even led to an increase in the number of pillar cells, but later these supporting cells underwent cell death. Compared to postnatal hair cells which undergo apoptosis immediately after cell cycle reactivation, *Rb* inactivated supporting cells seem to be able to progress further in the cell cycle and to avoid immediate death. Taken together, several studies using different approaches to inactivate *Rb* prove that pRb has an important role both during early embryonic development as well as in the postnatal period.

Table 1. Phenotypes of mutant mouse lines used in studies of the postmitotic state in the inner ear

mouse strain	additional info	phenotype	reference
p27^{Kip1} -/-	full *p27* ko	continued proliferation of PGs, proliferation of co SCs in adults, hearing loss (due to abnormal cellular organisation)	Chen and Segil 1999, Löwenheim et al. 1999
mgRb:Rb -/-	wt *Rb* minigene expressed only in nervous system (mice die at birth)	continued proliferation of PGs, proliferation of differentiated ve and co HCs, apoptosis and multinucleation observed	Mantela et al. 2005
Pou4f3-Cre:pRb$^{lox/lox}$	*Rb* deleted in ve HCs and co PGs	proliferation of co HCs and SCs, HC maturation defects, total co HC loss by 2 mo, proliferation of ve HCs, long-term survival of part of ve HCs	Sage et al. 2006
Atoh1-CreER:Rb$^{lox/lox}$	HC specific Cre line, TAM administration P0-P1	rapid cell cycle re-entry, no supernumerary HCs produced, immediate apoptosis	Weber et al. 2008
Prox1-CreER-Rb$^{flox/flox}$	SC (Deiter's and pillar cell) specific Cre line, TAM P0-P1	cell cycle re-entry of co SCs and progression in to mitosis, supernumerary pillar cells observed, apoptosis of SCs	Yu et al. 2010
ER-Cre-pRb$^{flox/flox}$	*Rb* inactivated at 3 mo by TAM	no proliferation of HCs or SCs	Huang et al. 2011
p19^{Ink4d} -/-	full *p19* ko	cell cycle re-entry of co HCs starting at 2nd postnatal week, followed by cell death, progressive hearing loss	Chen et al. 2003
p21^{Cip1}-/-	full *p21* ko	no effect on inner ear development or postmitotic state of HCs and SCs	Mantela et al. 2005
p27$^{lo/lo}$:CreER *(chapter 2.4.1)*	*p27* inactivated by TAM at 6 we	cell cycle re-entry of adult SCs (in co especially Hensen's cells), followed by apoptosis	Oesterle et al. 2011

PG=progenitor cell, SC=supporting cell, HC=hair cell, co=cochlear, ve=vestibular, ko=knock-out, TAM=tamoxifen, Rb =retinoblastoma, mo=months, we=weeks

p19^{Ink4d} is expressed in the organ of Corti starting at E14.5 (Chen et al. 2003). The inactivation of *p19^{Ink4d}* did not have an effect on the early development, cell cycle exit, or cellular differentiation in the organ or Corti (Chen et al. 2003). Instead, the cochlear hair cells of *p19^{Ink4d}* -/- mice re-entered the cell cycle starting at the 2nd postnatal week (see Table 1). Cell cycle re-entry was rapidly followed by cell death, which led to progressive hearing loss. This study showed that p19^{Ink4d} is involved in the maintenance of the postmitotic state of auditory hair cells (Chen et al. 2003). And similarly to neurons, differentiated hair cells that have their cell cycle re-activated quickly die apoptotically. The mechanism of hair cell death was not investigated in the study by Chen et al. (2003).

A third CKI expressed in the inner ear sensory epithelia is p21^{Cip1}. *p21^{Cip1}* expression was detected in embryonic and early postnatal vestibular and cochlear hair cells, and was induced at the initiation of hair cell differentiation (Mantela et al. 2005). In the auditory sensory epithelium, *p21^{Cip1}* expression was initiated at E14.5 at the stage when the differentiation marker Atoh1 is first detected (Chen et al. 2002). *p21^{Cip1}* induction in hair cells may be regulated by Atoh1 in a manner similar to skeletal myogenesis in which myogenic bHLH factor MyoD induces *p21^{Cip1}* expression (Guo et al. 1995, Halevy et al. 1995). Despite the specific expression pattern of *p21^{Cip1}* in the inner ear, the deletion of *p21^{Cip1}* did not have any effect on the inner ear development or the maintenance of the postmitotic state of hair cells (Mantela et al. 2005) (See Table 1). As the inner ear phenotype of *p19^{Ink4d}*-/- mice is mild and *p21^{Cip1}* deletion alone does not have an effect on inner ear, this suggests that these two, or other yet unknown cell cycle regulators might have redundant or co-operative roles in the regulation of the postmitotic state in the inner ear sensory epithelia.

2.4 Regeneration in the inner ear

Inner ear hair cells are extremely vulnerable to different kind of traumas, like noise exposure, aminoglycoside antibiotics, and cisplatin, a widely used chemotherapy drug. The adult mammalian inner ear lacks the plasticity that would allow natural hair cell regeneration. Thus, hair cell loss is a permanent event leading to hearing and balance deficits. This is in contrast to the ears of fish, amphibians and birds in which hair cells are replaced following trauma (Corwin and Cotanche 1988, Ryals and Rubel 1988). The regenerative potential of avian inner ear has been the focus of numerous studies. Thus, the avian cochlea and vestibular organs are probably the best understood biological models for hair cell recovery. Robust regeneration is observed in the avian cochlea (also called basilar papilla) after noise damage, and in both the cochlea and vestibular organs after aminoglycoside ototoxicity (Slattery and Warchol 2010). The latency of the regenerative response is very fast. Cell cycle entry in the basilar papilla occurs within 16 hours of hair cell injury (Warchol and Corwin 1996), and replacement hair cells can be observed within 2-3 days (Corwin and Cotanche 1988, Ryals and Rubel 1988, Duncan et al. 2006). Regeneration in the basilar papilla involves a combination of evoked proliferation and supporting cell transdifferentiation. The first replacement hair cells appear to arise directly from transdifferentiated supporting cells, while later recovery is due to supporting cell proliferation (Roberson et al 2004, Duncan et al. 2006). Importantly, functional studies on the regenerated avian ear have demonstrated that both hearing and vestibular sensitivities return to near-normal levels after sufficient recovery times (Bermingham-McDonogh and Rubel 2003).

As mentioned, the regenerative potential of the mammalian inner ear is very limited. Nevertheless, the vestibular organs of mammals retain a modest regenerative ability. Recovery of hair cells after ototoxic injury has been described in the vestibular organs of guinea pigs and mice (Forge et al. 1993, 1998, Kawamoto et al. 2009). Such recovery is much slower than that observed in the avian vestibular organs and typically requires 4-12 weeks before significant numbers of new hair cells are apparent (Forge et al. 1998). Even after much longer recovery periods, hair cell density never returns to normal levels. Quantification of the relative numbers of hair cells and supporting cells suggests that nearly all replacement hair cells are produced by the transdifferentiation of supporting cells (Forge et al. 1998). The vestibular organs of mammals also possess a very small number of supporting cells that have proliferative potential. After extensive ototoxic injury *in vitro*, a small but measurable number of proliferating supporting cells are observed in the mammalian utricle (Warchol et al. 1993, Lambert 1994).

2.4.1 Inducing regeneration

The lack of regenerative potential in the mammalian inner ear has inspired researchers for decades to search for different approaches to induce regeneration. One approach that is under investigation is the use of stem cells, either embryonic (ES) or induced pluripotent stem (iPS) cells, as a starting material to generate new, functional hair cells and supporting cells. A stepwise guidance protocol for generating mechanosensitive hair cell-like cells from ES and iPS cells has been successfully created (Oshima et al. 2010). However, there are still several obstacles on the way to real regeneration. One of the biggest obstacles is the delivery and integration of cells produced *in vitro* on a culturing plate into the inner ear. Another approach is to induce regeneration by manipulating existing sensory epithelial cells, either *in vitro* or preferably *in vivo*. If we consider those properties of a cell that most probably would influence its regenerative capabilities, the cell cycle status and cellular morphology play certainly an important role. Several studies have focused on studying and manipulating these properties in order to reveal the underlying causes for defective regeneration in mammals.

The cellular morphology of the sensory epithelium of the avian basilar papilla and the mammalian organ of Corti are very different. The sensory epithelium of the avian basilar papilla resembles that of the vestibular organs, and is comprised of hair cells that are surrounded by relatively undifferentiated supporting cells. In contrast, the mammalian organ of Corti contains highly specialised supporting cell phenotypes, which are different both at the morphological and the molecular level. The structural complexity of the organ of Corti, at the level of hair cells and supporting cells, probably evolved in order to permit sensitive high frequency hearing (Dallos 2008). In this view, the lack of cochlear regeneration might be explained as the result of a trade-off between structural complexity and phenotypic plasticity. Recent studies have revealed that the cytoskeletal structures of cell-cell junctions in the avian and mammalian vestibular sensory epithelia are very different (Burns et al. 2008). Burns et al. (2008) found that circumferential F-actin bands in utricular supporting cells grow much thicker as mice and humans mature postnatally, whereas their counterparts in chicken remain thin until adulthood. The steepest accumulation of F-actin occurs coincidentally with early postnatal decline in the capacity for supporting cells to change their shape from columnar to spread shape and proliferate near sites of injury *in vitro* (Davies et

al. 2007, Burns et al. 2008). Avian supporting cells respond to injury to the epithelium with high levels of spreading and proliferation that do not decline with age. These studies implicate that the presence of thin F-actin bands at the junctions between avian supporting cells may contribute to the lifelong persistence of their capacity for shape change, cell proliferation, and hair cell replacement (Davies et al. 2007, Burns et al. 2008). This indicates that the manipulation of cytoskeletal and cell-cell junctional structures could possibly be one avenue to enhance regeneration in the mammalian sensory epithelium.

It is likely that the restrictions in the regenerative capacity of the mammalian inner ear are also related to processes other than those regulating cellular morphology. In order to achieve regeneration, proliferation of either supporting cells or remaining hair cells is needed. To induce proliferation in normally postmitotic cells, it is required that pathways leading to cell cycle progression are activated and those guarding the postmitotic state are inactivated. Several studies have focused on finding out whether postmitotic sensory epithelial cells naturally have proliferative potential and whether this potential could be enhanced by different manipulations. As mentioned earlier, the inactivation of Rb in early postnatal hair cell or supporting cells induces cell cycle re-entry (Weber et al. 2008, Yu et al. 2010), but a similar response was not observed in adults (Huang et al. 2011). These studies clearly show that there are age-related differences in the maintenance of the postmitotic state. Several studies have investigated the response of vestibular supporting cells to exogenous mitogens, such as EGF, FGF and GGF, *in vitro* (Zheng et al. 1997, Montcouquiol and Corwin 2001a, 2001b, Hume et al. 2003, Gu et al. 2007, Lu and Corwin 2008). These studies show that vestibular supporting cells can re-enter the cell cycle in response to a mitogenic stimulus neonatally, but this capacity is almost completely lost at later stages (Gu et al. 2007). The steepest decline in the response to mitogens occurs around postnatal day 5 (P5) (Gu et al. 2007). Despite the testing of extensive numbers of known growth factors by several groups, factors capable of stimulating proliferation in the mammalian auditory epithelium, the organ of Corti, have not yet been identified.

As $p27^{Kip1}$ is an important cell cycle regulator in supporting cells, the difference in regenerative capacity between mammals and birds may be due to the $p27^{Kip1}$ status in adult supporting cells: either $p27^{Kip1}$ levels in mammals are too high or they are inefficiently downregulated after injury. The decreased $p27^{Kip1}$ levels in the supporting cells could allow the cells to re-enter the cell cycle. White et al. (2006) showed that when supporting cells ($p27^{Kip1}$+) are purified from neonatal (P3) cochlea and cultured together with periotic mesenchymal cells they form small epithelial islands and within 2 DIV downregulate $p27^{Kip1}$ and enter the S-phase. Few days later, cells positive for myosin VI, a specific hair cell marker, were observed in these islands. Many of these new hair cells were BrdU+, but some also BrdU- indicating that the transdifferentiation of supporting cells into hair cells had happened both through mitotic and non-mitotic mechanisms. When the same experiment was conducted with supporting cells purified from functionally mature cochlea at P14, only very few supporting cells re-entering the cell cycle were observed (2%) and the rest of the cells continued to express $p27^{Kip1}$. When supporting cells purified from *$p27^{Kip1}$* null mice were used, the number of BrdU+ cells was significantly higher compared to wild-type counterparts. This indicates that the ability to downregulate $p27^{Kip1}$ is a key mechanism in the cell cycle re-entry of supporting cells. It has been shown also in explant culture experiments that silencing of *$p27^{Kip1}$*

expression by siRNA stimulates BrdU incorporation in neonatal cochlear supporting cells, indicating S phase activity, and that these cells can progress into mitosis (Ono et al. 2009). Another recent study used an *in vivo* approach to study the proliferative potential of adult supporting cells in response to inducible *p27^{Kip1}* inactivation (Oesterle et al. 2011) (see Table 1). This study showed that adult (6 week-old) supporting cells are able to re-enter the cell cycle in response to *p27^{Kip1}* inactivation, but the level of proliferation in the adult supporting cells was greatly reduced when compared to neonates (P7). According to this study it also appears that there are differences among the supporting cell subtypes in their response to p27^{Kip1} inactivation, such that in the neonates the highest proliferative activity was seen in pillar cells and in adults the highest activity was in Hensen's cells. These differences cannot simply be explained by the p27^{Kip1}expression patterns, suggesting that additional mechanisms are required to regulate supporting cell quiescence.

Several studies have shown that despite the lack of natural regenerative capacity in the mammalian inner ear, differentiated supporting cells still have some proliferative potential, even if it seems that adult supporting cells are less plastic than neonatal ones. However, several questions about the proliferative potential of these cells still remain. Many of the studies conducted during recent years have used BrdU labelling as a readout of proliferative activity rather than looking for further progression of the cell cycle. But if we consider the induction of cell cycle re-entry as a way to induce real regeneration, the completion of the cell cycle and production of progeny has to be taken into account. In addition, deeper knowledge about the components of the basic cell cycle machinery controlling the postmitotic state of hair cells and supporting cells is required.

3. AIMS OF THE STUDY

As mammalian hair cells and supporting cells are not able to regenerate, understanding the maintenance of their postmitotic state is likely to be one avenue that can take us closer in finding ways to induce regeneration in the mammalian inner ear.

The specific aims of this study were:

1. To study the maintenance of the postmitotic state of hair cells during postnatal life. Specifically, to study the involvement of two CKIs, $p19^{Ink4d}$ and $p21^{Cip1}$, in the maintenance of the postmitotic state.

2. To study the role of cyclin D1 in the cell cycle regulation of hair cells and supporting cells. To investigate the possible co-operative role of CKIs and cyclin D1 in the maintenance of the postmitotic state.

3. To study the proliferative potential of mature supporting cells and the response of these cells to ectopic cyclin D1 expression.

4. MATERIALS AND METHODS

Mouse strains, probes, antibodies and methods used in this study are listed in tables below. The detailed information can be found in the articles I-III (as indicated in each case).

4.1 Mouse strains

mouse strain	used in article	purpose	reference
NMRI	I, II, III	mRNA and protein expression studies, *in vitro* cultures	
$p19^{Ink4d}$ -/-	I	analysis of *$p19^{Ink4d}$* deficient phenotype	Zindy et al. 2000, Chen et al. 2003
$p21^{Cip1}$ -/-	I,III	analysis of *$p21^{Cip1}$* deficient phenotype, analysis of involvement of $p21^{Cip1}$ in G2/M checkpoint	Brugarolas et al. 1995
$p19^{Ink4d}/p21^{Cip1}$ -/- (dko)	I, II	analysis of dko phenotype, AdcD1 misexpression studies	I
Math1-GFP	I	purification of hair cells by FACS (for quantitative PCR)	Doetzlhofer et al. 2004
BAT-gal	II	Wnt/β-catenin signalling studies	Maretto et al. 2003

4.2 Probes

probe	used in article	reference
p15Ink4b	II	Zindy et al. 1997
p16Ink4a	II	Zindy et al. 1997
p18Ink4c	II	Zindy et al. 1997
p19Ink4d	II	Zindy et al. 1997
p21Cip1	II,III	El Deiry et al. 1993
p27Kip1	III	Park et al. 1999
p57Kip2	II	Matsuoka et al. 1995
cyclin D1	II	Trokovic et al. 2005

4.3 Antibodies

antibody	host	used in article	source/reference	cat.#
phospho-ATM	mouse	I	Millipore	05-740
Aurora B	rabbit	III	Sigma	A5102
β-galactosidase	mouse	II,III	Promega	Z378A
β-catenin	mouse	II	BD Biosciences	610153
BrdU	mouse	III	LabVision/Thermo Scientific	MS-949-P
cyclin E	rabbit	I	Millipore	06-459
cyclin D1	rabbit	I,II,III	LabVision/Thermo Scientific	RM-9104
cyclin D2	mouse	II	LabVision/Thermo Scientific	MS-221
phospho-Chk2	rabbit	I	Cell Signalling Technology	2661
cleaved caspase 3	rabbit	I,III	Cell Signalling Technology	9664
E-cadherin	rat	III	Sigma	U3254
phospho-H2AX	mouse	I,III	Millipore	05-636
phospho-H2AX	rabbit	I	Millipore	07-164
Ki-67	mouse	I,II,III	Novocastra	NCL-Ki67-MM1
Ki-67	rabbit	I,II,III	LabVision/Thermo Scientific	RM-9106
myosin VIIa	rabbit	I,II	Hasson et al. 1997	
parvalbumin	goat	II,III	Swant	PVG-214
PCNA	mouse	I	Novocastra	NCL-PCNA
phospho-PH3	mouse	III	Cell Signalling Technology	9706
phospho-PH3	rabbit	I,III	Cell Signalling Technology	9701
p19Arf	rat	I	Millipore	05-929
p21	mouse	III	BD Biosciences	556431
p27	mouse	III	BD Biosciences	610241
p27	mouse	II	LabVision/Thermo Scientific	MS-256
p57	goat	II	Santa Cruz Biotechnology	SC-1039
p53	rabbit	I	Novocastra	NCL-p53-CM5p
phospho-p53	rabbit	I	Cell Signalling Technology	9284
Prox1	rabbit	I	Covance	11-002
Sox2	goat	III	Santa Cruz Biotechnology	SC- 17320
Sox9	rabbit	III	Millipore	AB5535

4.4 Methods

method	used in article
Genotyping	I,II,III
Histology and immunohistochemistry on paraffin sections	I,II,III
Histology and immunohistochemistry for sensory epithelial whole-mounts	I,II,III
Organotypic cultures (see also chapter 4.4.1)	II,III
Adenoviral infections (see also chapter 4.4.1)	II,III
Radioactive in situ hybridization on paraffin sections	II,III
Cochleograms	I
Semithin sections	I,III
β-Galactosidase staining	II
Hair cell purification by FACS and quantitative PCR	I
Transfection of HeLa cells	I
Labeling with thymidine analogs	III
Apoptosis detection with ApopTag Plus Fluorescein *In Situ* Apoptosis Detection Kit	III

4.4.1 Organotypic cultures and adenoviral infections

In this study (especially in articles II and III), the establishment of organotypic cultures of the utricular sensory epithelium and their infection with adenoviruses was one of the key methods. In this chapter these methods will be described in more detail.

4.4.4.1 Establishment of organotypic cultures

To establish organotypic culture, the utricular sensory epithelium was first dissected out from the inner ear by using fine forceps and needles under sterile conditions. After dissection, the utricular explant was placed on a piece of Nucleopore filter membrane (Whatman), which was placed on a metal grid in Dulbecco's modified Eagle's medium/F-12 medium supplied with 2 mM L-glutamine and penicillin (100 U/mL) (Gibco/Invitrogen) and 10% fetal bovine serum (FBS) (HyClone/Thermo Scientific). Explants were stabilized on filters for 12 hours allowing them to attach properly on the filter membrane. Incubations were done in a humidified 5% CO_2 atmosphere at 37°C (Fig. 4).

4.4.4.2 Adenoviral infections

In these studies we used adenoviral vectors of serotype 5. Transgenes used were under the *CMV* (cytomegalovirus) promoter. Cloning and propagation of these recombinant viruses has been described previously (Albrecht and Hansen 1999). Adenoviruses (serotype 5) infect inner ear sensory epithelial cells with high efficiency (Kirjavainen et al. 2008). Tropism of these viruses towards hair cells and supporting cells changes dramatically during the early postnatal maturation period. Prior *in vitro* studies have shown that hair cells are the main cell type in the inner ear sensory epithelia infected by adenoviruses at late-embryogenesis and at birth, and that hair cells gradually become refractory to these viruses during the first and second postnatal weeks (Kirjavainen et al. 2008). In the paper II, these viral vectors were used to infect utricular hair cells at P5, and in the paper III, the same viruses were used to target adult utricular (P50) supporting cells.

After a 12-hour long stabilization period, utricular explants were infected with adenoviral vectors. Infections were done in 25 µl drops of serum-free culturing medium (P5 utricles) or

culturing medium containing 2% of FBS (adult utricles) on sterile culture plates. Viral concentrations were 1.93-2.0 x 10^7 pfu/ml for AdβGal (adenovirus carrying the β-galactosidase gene) and 1.97-2.3 x 10^7 pfu/ml for AdcD1 (adenovirus carrying the cyclin D1 gene). Explants (attached on the filters) were placed into the drops and maintained there for 6 to 8 hours. After infection, explants were returned to metallic grids and normal culturing media (10% FBS) for additional 3 to 12 days (Fig. 4). The culture medium was changed every other day. After culturing, explants were fixed with 4% PFA (paraformaldehyde) in PBS and stained according to the whole-mount immunohistochemistry protocol (see protocol in papers I-III).

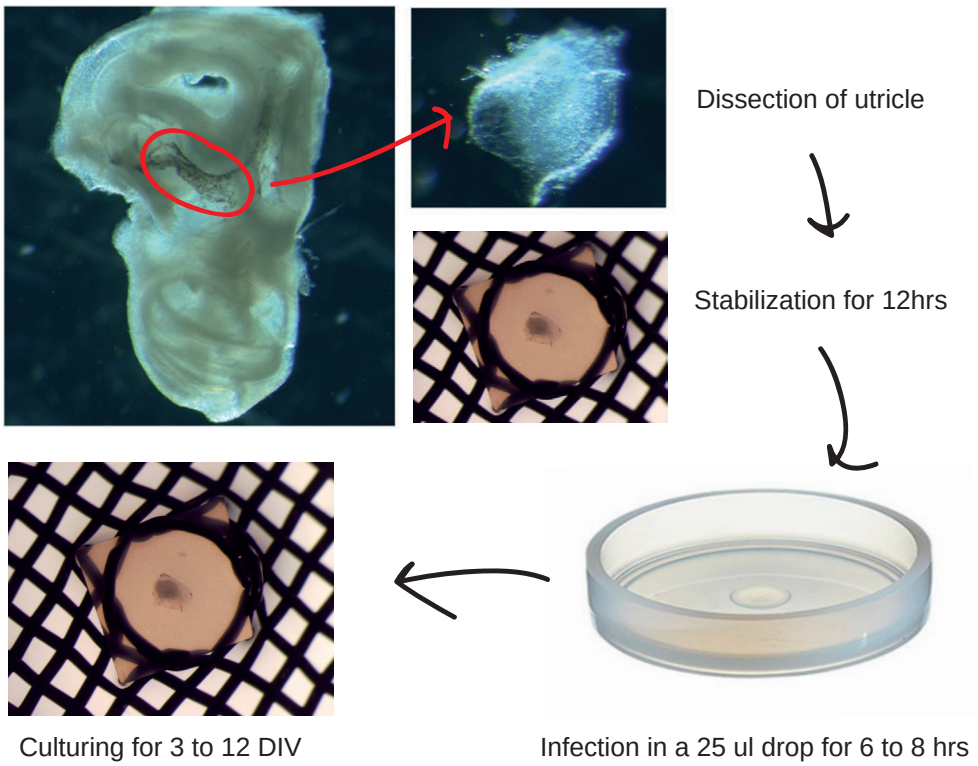

Figure 4. The establishment of organotypic culture and adenoviral infection. First, the utricular sensory epithelium is dissected out from the inner ear. The red oval shows the location of the utricle. When the epithelium is dissected out, it is placed on a filter membrane on a metal grid. The metal grid is in a culturing plate with culture medium containing 10% FBS. Utricular explants are stabilized for 12 hours in an incubator. A drop of medium containing the adenovirus is pipetted to culturing plate and the explant (attached on the filter) is placed into the drop and kept there for 6 to 8 hours. After infection, culturing is continued for 3 to 12 days on a grid.

5. RESULTS AND DISCUSSION

5.1 p19^{Ink4d} and p21^{Cip1} collaborate to maintain the postmitotic state of auditory hair cells (I)

Maintenance of the postmitotic state of auditory hair cells has been shown to require p19^{Ink4d} (Chen et al. 2003). In the study by Chen et al. (2003) it was shown that the inactivation of *p19^{Ink4d}* leads to cell cycle re-activation in postnatal hair cells. Cell cycle re-entry began at the end of the first postnatal week and progressed slowly. Hair cell proliferation was followed by apoptosis, which led to progressive hearing loss. In contrast, the loss of *Rb* led to an apparent complete loss of control of the postmitotic state of hair cells, which resulted in severe hyperplasia in the organ of Corti (Mantela et al. 2005, Sage et al. 2005, 2006). The more extreme loss of control of the postmitotic state observed in the *Rb* mutant mice compared with the *p19^{Ink4d}* -/- mice suggested that additional CKIs may be involved in the maintenance of the postmitotic state of hair cells. It was shown earlier that p21^{Cip1} is expressed in hair cells, but its inactivation did not lead to an altered hair cell phenotype (Mantela et al. 2005). Previous data on *p19^{Ink4d}* and *Rb* mutant phenotypes and CKI expression patterns led us to hypothesize that p19^{Ink4d} and p21^{Cip1} might act in collaboration to control the postmitotic state of hair cells. A similar phenomenon has been shown in several other cell types (Zindy et al. 1999, Cunningham et al. 2002), where CKIs act redundantly to inhibit proliferation. To test this hypothesis, we generated a mouse line where *p19^{Ink4d}* and *p21^{Cip1}* are simultaneously inactivated (hereafter termed dko=double knock-out).

The dko mice were viable and the gross morphology of the inner ear was not affected by the codeletion of these two CKIs (analyzed from E16.5 to adulthood) (Publication I/Fig. 1). When analyzed at birth, the cellular arrangement in the organ of Corti was comparable between dko and wild type mice. At that time point, hair cells were postmitotic nt S-phase entry compared to OHCs (outer hair cells) (I/Fig. 2). Mitotic figures (PH3 staining and semithin sections) were mainly seen in IHC area (I/Fig. 3). Overall, the numbers of PCNA+ hair cells clearly surpassed those of mitotic hair cells. Excess IHCs were observed indicating successful divisions (I/Fig. 3). Some IHCs showed an abnormal nuclear morphology indicating problems in cytokinesis. The organ of Corti of the dko mice displayed a normal complement of supporting cells (I/Fig. 3). The lack of S-phase and mitotic markers in supporting cells indicated that the codeletion of *p19^{Ink4d}* and *p21^{Cip1}* did not affect the postmitotic state of cochlear supporting cells (I/Fig. 2 and 3). The first signs of hair cell loss in the organ of Corti were seen at P6 (2.5% of IHCs and 5% of OHCs lost), and the loss rapidly progressed thereafter (I/Fig. 4). We observed hair cells positive for cleaved caspase 3, as well as apoptotic nuclear morphology in semithin sections, indicating that soon after cell cycle re-entry hair cells died through apoptosis (I/Fig. 3). This observation is consistent with the previous data on the apoptotic death of auditory hair cells from *p19^{Ink4d}* (Chen et al. 2003) and *Rb* (Mantela et al. 2005) mutant mice. Chen et al. (2003) reported progressive hair cell loss in *p19^{Ink4d}* mutants: no hair cell loss was observed at P5, but by 7 weeks of age, a loss of 43% of IHCs and 15% of OHCs was found in the basal region of the cochlea. In that study, hair cell loss in the other regions of the cochlea was not studied. We counted the hair cell loss throughout the length of the cochlear

duct in two month-old *p19*^{Ink4d} mutants and dkos (I/Fig. 4). Consistent with Chen et al. (2003), we found that the basal and middle regions of the cochlea were most severely affected in the *p19*^{Ink4d} mutant, with the loss of 29% of IHCs and 9% of OHCs (0-4mm from the base of the cochlea). Only minor hair cell loss was seen at the upper levels of the cochlea. The dko mice showed significant loss of IHCs and OHCs throughout the length of the cochlear duct. Middle and basal regions were most severely affected, with the loss of 66% of IHCs and 87% of OHCs in the middle region, and 64% of IHCs and 62% of OHCs in the basal region. The best cellular preservation was seen in the apical region. These results clearly showed that the simultaneous inactivation of *p19*^{Ink4d} and *p21*^{Cip1} has a much stronger effect on hair cells than the inactivation of either factor alone.

As we showed that the codeletion of *p19*^{Ink4d} and *p21*^{Cip} led to S-phase entry of auditory hair cells at a very precise time window in early postnatal life, we wanted to investigate the possible redundancy among other CKIs. The expression of different CKIs in auditory hair cells was studied by quantitative-PCR in late embryogenesis (E17) and during the first postnatal week (at P0, P2, P4 and P7) (I/Fig. 5). Q-PCR results indicated that the decreasing levels of *p57*^{Kip2} between P0 and P4, and the rising levels of *p27*^{Kip1} at P7 might help to explain the short time of aberrant hair cell proliferation seen in dko mice (I/Fig. 5). Thus, when both *p21*^{Cip1}and *p19*^{Ink4d} are deleted, the fluctuating expressions of the remaining CKIs could make the hair cells variably susceptible to disruptions of the postmitotic state at different time points.

p53 has been indicated to be a mediator of cell death following different kinds of cellular stresses, like abnormal proliferation (Vousden and Lu 2002). As hair cell death following abnormal cell cycle re-entry had not been studied previously in detail, we wanted to investigate the mechanisms and molecular pathways behind the apoptosis of hair cells with abnormal proliferation. We showed that abnormally proliferating hair cells experience replicative stress that leads to the formation of double-strand breaks (DSBs) in DNA (I/Fig. 6). The presence of DSBs in hair cells was detected by staining with phospho-H2AX antibody, which clearly marks the foci of DSBs. Next, we tested whether other members of the DNA damage response (DDR) pathway are activated in hair cells by staining with antibodies against phospho-ATM, phospho-Chk2, and phospho-p53. Hair cells of dko mice at P6 stage reacted to all of these markers of DDR pathway (I/Fig. 6). In contrast, the hair cells of wild-type mice did not express any of these markers (I/Fig. 6). This suggests that S-phase entry of hair cells triggers the activation of the DDR pathway, which in turn results in the activation of p53 and finally to apoptosis. This suggestion is supported by previous *in vitro* data showing that p53 is activated in hair cells exposed to cisplatin, a DNA-damaging drug (Zhang et al. 2003). Protection from cisplatin-induced apoptosis was achieved by using a p53 inhibitor pifithrin-α. Furthermore, a more recent study showed that *p53* inactivation attenuates hair cell loss triggered by cell cycle re-activation (Sulg et al. 2010)

It is well known that p21^{Cip1} has versatile functions in the cell cycle compared to other CKIs. In addition to the regulation of cell cycle progression by binding to different Cdk/cyclin complexes, p21^{Cip1} is an important p53-inducible effector of the G2/M checkpoint. In response to DNA damage or other types of cellular stresses, p53 can induce cell cycle arrest by activating p21^{Cip1} expression. In the absence of p21^{Cip1}, some proliferating cells can escape cell cycle arrest even if they experience DNA damage (Bunz et al. 1998, Niculescu et al. 1998). The lack of p21^{Cip1} may explain why part of the auditory hair cells of the dkos were able to progress into mitosis despite

the DNA damage. Nevertheless, $p21^{Cip1}$ is not likely to be the sole factor regulating G2/M arrest, as infrequent mitoses were also observed in *p19*^{Ink4d} mutants (unpublished data). Mitotic abnormalities in dko samples were detected in semithin sections, which suggests that hair cells that entered mitosis with DNA damage had problems in later phases of mitosis and cytokinesis. It was mainly IHCs that entered mitosis, and even some completed it, indicating that there may be differences in the checkpoint control between IHCs and OHCs. Based on these results, we cannot rule out the possibility that the cells entering mitosis were originally DNA damaged but were able to repair it. However, this is an unlikely situation as DNA damage repair capability of highly differentiated postmitotic cells is in general very poor. The overall loss of hair cells in adult dkos was similar in IHCs and OHCs ($P>0.05$), indicating that hair cell loss was not related to cell cycle progression and that hair cell death probably took place at different phases of the cell cycle. The fact that IHCs were able to progress further in cell cycle is in accordance with the previous studies showing that IHCs are more resistant to several types of environmental stressors compared to OHCs (Cardinaal et al. 2000). Furthermore, a recent study from our group (Sulg et al. 2010) showed that OHCs are more sensitive to elevated p53 levels than IHCs. Thus, the sensitivity to p53 levels might explain why OHCs were not able to progress in the cell cycle and were lost more rapidly compared to IHCs in the dko-model.

Together with the notion that S-phase re-entry of auditory hair cells was restricted to a very limited time window in early postnatal life, one of the most interesting findings of this study was that vestibular hair cells did not react to codeletion of *p19*^{Ink4d} and *p21*^{Cip1} (I/Fig. 8). This suggests that there are certain additional differences in the control of the postmitotic state between vestibular and cochlear hair cells. Although the actively changing levels of other CKIs expressed in the hair cells may help to explain the abrupt onset of cell cycle entry seen in dkos, it still remains unclear whether this redundancy functionally exists. It also raises a question about the possible involvement of other cell cycle regulatory proteins in the maintenance of the postmitotic state of inner ear hair cells. The molecular mechanisms controlling the postmitotic state in the postnatal vestibular sensory epithelia are even more poorly understood and the lack of cell cycle re-entry in the vestibular sensory epithelia in response to inactivation of both *p19*^{Ink4d} and *p21*^{Cip1} underlined the need to examine this aspect more carefully.

5.2 Cyclin D1 is involved in the cell cycle regulation in the inner ear sensory epithelia (II)

Inspired by the distinct spatiotemporal pattern of cell cycle re-entry after inactivation of *p19*^{Ink4d} and *p21*^{Cip}, we aimed to identify additional factors that regulate the maintenance of the postmitotic state in the inner ear sensory epithelia.

First, we studied the detailed spatial expression patterns of different CKIs during the postnatal period (II/Fig. 1 and 2) because in the previous qPCR screen (I/Fig. 5), we could not map the detailed expression and see the possible differences in the expression patterns between IHCs and OHCs or different regions of the cochlear duct. We found that *p19*^{Ink4d} was very strongly and specifically expressed in all hair cells both in the cochlea and in the vestibular sensory epithelia (II/Fig. 1). Other INK4 family members were not expressed in the inner ear sensory epithelia, confirming our previous qPCR results (II/Fig. 1). Compared to *p19*^{Ink4d}, *p21*^{Cip} expression was

more widespread comprising both supporting cells and hair cells in all sensory epithelia (II/Fig. 1). *p27^{Kip1}* is known to be expressed in supporting cells of the embryonic and early postnatal inner ear sensory epithelia (Chen and Segil 1999, Löwenheim et al. 1999, Lee et al. 2006, White et al. 2006). We found that p27^{Kip1} is prominently expressed in cochlear supporting cells from birth until adulthood, with the exception of Deiter's cells which expressed p27^{Kip1} only weakly in the adulthood (II/Fig. 2). Supporting cells of the vestibular organs expressed p27^{Kip1} throughout postnatal life (II/Fig. 2). In contrast to cochlear hair cells, we found p27^{Kip1} expression also in a small population of utricular hair cells located in a scattered manner within the epithelium (II/Fig. 2). p57^{Kip2}, the third member of the Cip/Kip subfamily, was expressed in cochlear hair cells early postnatally, but became downregulated by P3 (II/Fig. 2). This detailed expression study confirmed that the differences in the ability of different populations of the inner ear hair cells from the *p19^{Ink4d}/p21^{Cip1}* dko mice to re-enter the cell cycle can not be fully explained by the CKI expression patterns. Most strikingly, we detected the expression of *p19^{Ink4d}* and *p21^{Cip1}* both in cochlear and vestibular hair cells, but their codeletion led to cell cycle re-activation only in cochlear hair cells. Furthermore, CKI expression patterns are relatively homogenous along the length of the cochlear duct, and thus, are not consistent with the spatially restricted pattern of abnormal cochlear hair cell proliferation seen in the dko mice.

D-type cyclins are essential in the cell cycle control. They are induced by mitogens and control the inactivation of Rb, and thus, trigger commitment to a new round of cell division. Several studies have shown that during a restricted early postnatal period, vestibular supporting cells are able to respond to exogenous mitogenic cues (Zheng et al. 1997, Montcouquiol and Corwin 2001a, 2001b, Hume et al. 2003, Gu et al. 2007, Lu and Corwin 2008). We wanted to study whether D-type cyclins are expressed in the inner ear and do they contribute to the regulation of proliferative potential of different cell populations in the inner ear sensory epithelia. We found that cyclin D1 (cD1) has an interesting expression pattern in the inner ear. Widespread expression of cD1 can be seen during early embryogenesis throughout the developing sensory epithelia (II/Fig. 6). When cellular differentiation starts, cD1 expression is downregulated in hair cells (both in the vestibular and cochlear sensory epithelia) (II/Fig. 6). All supporting cells, except Deiter's cells in the cochlea, continue to express cD1 at birth (II/Fig. 4). Interestingly, cD1 shows a transient upregulation in cochlear hair cells during early postnatal life (II/Fig. 4). This upregulation was first seen in the upper basal part of the cochlea at P2. By P4, cD1 expression had expanded into the middle part of the cochlea, but the apical part remained negative. At P7, cD1 was still localized to hair cells at the middle and upper basal parts of the cochlea. Thereafter, the expression of cD1 was downregulated, so that at P10 several IHCs but only few OHCs were positive. At P15 and thereafter, cD1 was undetectable in cochlear hair cells. Following the same temporal pattern, cD1 was also downregulated in cochlear supporting cells (II/Fig. 4). In the vestibular sensory epithelia, cD1 expression was strong in supporting cells at birth, but thereafter progressively disappeared from these cells. Already at P7, a large part of vestibular supporting cells were negative for cD1, and at adulthood only a very small part of supporting cells continued to express cD1 (II/Fig. 4). This data showed that the transient expression of cD1 in cochlear hair cells precisely corresponds to the pattern of abnormal hair cell proliferation seen in the *p19^{Ink4d}/p21^{Cip1}* dko mice (II/Fig. 3 and 4). Furthermore, the lack of cD1 expression in the vestibular hair cells could explain the absence

of proliferative activity in these cells following the inactivation of $p19^{Ink4d}$ and $p21^{Cip1}$. To directly address the hypothesis that cD1 expression underlies the pattern of unscheduled proliferation of hair cells in dko mice, we misexpressed cD1 *in vitro* in utricular hair cells of wild type and dko mice by using adenoviral-mediated gene transfer approach. The results of this experiment confirmed our hypothesis: only hair cells from dko mice were able to re-enter the cell cycle following cD1 misexpression (II/Fig. 5). Taken together, cD1 overexpression can trigger cell cycle re-entry only in the absence of CKIs, suggesting that the maintenance of the postmitotic state of hair cells requires tight control of the expression of both positive and negative cell cycle regulators.

cD1 has a very dynamic expression pattern during early postnatal life. But what is the function of this specific expression profile? The time period when cD1 is transiently expressed in cochlear hair cells corresponds to the period of functional maturation of these cells. During this maturation period cochlear hair cells have also been reported to be hypersensitive to different kind of environmental traumas (Henley and Rybak 1995). In neurons cD1 has been shown to function as a proapoptotic factor in response to trauma (Ino and Chiba 2001). Whether cD1 is involved in the regulation of apoptotic response in hair cells still remains to be solved. Supporting cells of the cochlea (except Deiter's cells) and vestibular organs showed strong expression of cD1 at birth, but this expression rapidly declined thereafter. The expression of cD1 in utricular supporting cells corresponds to the time period when these cells have capacity to proliferate in response to mitogens. Thus, it seems likely that cD1 expression in supporting cells is required for their response to mitogens. The assumption that cD1 expression is critical for the proliferative capacity of utricular supporting cells is supported by the reported behaviour of cochlear supporting cells. White et al. (2006) showed that supporting cells purified from neonatal cochlea and maintained *in vitro* can re-enter the cell cycle and that this event is associated with $p27^{Kip1}$ downregulation. When supporting cells purified from P14 cochlea were used, cell cycle re-entry was not observed. Thus, this study reported two important phenomena: first, cell cycle re-entry is clearly restricted to early postnatal stages (as cD1 expression also indicates) and secondly, supporting cells are capable of downregulating $p27^{Kip1}$ in order to achieve cell cycle re-entry. The notion of $p27^{Kip1}$ downregulation is of great importance, as it shows that supporting cells have an intrinsic potential to downregulate CKI expression when these cells are induced to re-enter the cell cycle. In contrast, ectopic cD1 expression in hair cells could induce cell cycle re-entry only in the absence of $p19^{Ink4d}$ and $p21^{Cip1}$ (in dko) but not in the wild type.

The distinct spatiotemporal pattern of cD1 expression in inner ear also raised the question of its regulation. cD1 is known to be one of the many targets of the canonical Wnt/β-catenin pathway (Tetsu and McCormick 1999, Shtutman et al. 1999). We studied the activity of Wnt/β-catenin signalling *in vivo* using *BAT-gal* reporter mice that express LacZ under the control of the transcription factor *Tcf/Lef* binding sites (Maretto et al. 2003). We did not find a correlation between *BAT-gal* activity and the distribution of cD1-positive cells (II/Fig. 7). We further investigated Wnt/β-catenin pathway *in vitro* by treating cochlear cultures from P0 mice with BIO. BIO is a specific GSK-3 (glycogen synthase kinase 3) inhibitor (Meijer et al. 2003). In the absence of Wnts, GSK-3 directs β-catenin to proteosomal degradation (Clevers 2006). When BIO was applied to cochlear cultures, β-catenin accumulated to the cell nuclei, including hair cell nuclei. However,

the accumulation of β-catenin did not have any effect on cD1 expression. These experiments suggested that cD1 is not a target of Wnt/β-catenin signalling in the cochlear sensory epithelium. Therefore, revealing the upstream regulators of cD1 awaits future studies.

5.3 Cyclin D1 and the proliferative potential of adult supporting cells (III)

There are several reasons to think that supporting cells would be better candidates than hair cells for the source of regeneration in the inner ear. Supporting cells have naturally better proliferative potential compared to hair cells, and supporting cells are able to respond to mitogenic cues, at least during early postnatal period. In addition, hair cells do not tolerate cell cycle re-entry; following cell cycle re-entry, they quickly die apoptotically. As our earlier studies (II) showed that cD1 downregulation in vestibular supporting cells closely parallels the stage when these cells show a steep decline in mitogen responsiveness, we wanted to study if ectopically expressed cD1 would be capable to force mature supporting cells back into the cell cycle. In addition, we wanted to investigate whether adult supporting cells tolerate cell cycle re-entry. We addressed these questions by using adenoviruses as tools to express cD1 (AdcD1) in mature supporting cells of the utricle (one of the vestibular sensory epithelia) *in vitro* in an explant culture system. Prior *in vitro* studies have shown that during late embryogenesis and the early postnatal period, hair cells are the main cell type in the inner ear infected with adenoviruses (serotype 5) (II, Kirjavainen et al. 2008). However, hair cells become refractory to these viruses gradually during the two first postnatal weeks (II, Kirjavainen et al. 2008). We found that supporting cells have the opposite profile of tropisms. And thus, in the mature utricle, supporting cells are infected with high efficiency (over 50%) (III/Fig. 1). Mature supporting cells survive well in long-term culture conditions. This is in contrast to mature hair cells which are rapidly lost after a few days in culture (Quint et al. 1998) (III/Fig. 1). The fact that supporting cells tolerate *in vitro* conditions well and that they are infected with adenoviruses with high efficiency makes our culturing system a suitable model to study the effect of ectopic cD1 expression in mature supporting cells.

We found that ectopically expressed cD1 triggers robust cell cycle re-entry of adult utricular supporting cells (III/Fig. 2). On average, 26.4% of supporting cells were Ki-67+ (marks G1, S, G2, and M phases of the cell cycle) at 7DIV. As mentioned before, $p27^{Kip1}$ is important CKI for the regulation of the postmitotic state of supporting cells. In control cultures (infected with AdβGal), $p27^{Kip1}$ was uniformly expressed in all supporting cells (III/Fig. 3). In AdcD1 infected cultures $p27^{Kip1}$ was suppressed in part of the supporting cells. Co-staining with $p27^{Kip1}$ and Ki-67 revealed that $p27^{Kip1}$ suppression was confined to the Ki-67+ population of supporting cells (III/Fig. 3). In the study by White et al. (2006), it was reported that neonatal cochlear supporting cells were able to downregulate $p27^{Kip1}$ and re-enter the cell cycle when purified and cultured *in vitro*. They also showed that this ability was lost by the second postnatal week. According to our studies, cD1 is strongly expressed in neonatal cochlear supporting cells (except Deiter's cells), but is lost almost completely by P15 (II/Fig. 4). Taken together, this indicates that supporting cells require cD1 to be able to downregulate $p27^{Kip1}$, and thus, to re-enter the cell cycle. Whether $p27^{Kip1}$ downregulation is achieved by direct interaction between $p27^{Kip1}$ and cD1, or whether it is achieved by other means that are not directly dependent on cD1 expression, but rather depend on other changes

that take place upon cell cycle entry is not clear. In contrast to $p27^{Kip1}$, $p21^{Cip1}$ expression became upregulated in the supporting cells that had re-entered the cell cycle (III/Fig. 4). $p21^{Cip1}$ is one of the many targets in various cell cycle checkpoints, and thus, its upregulation can indicate the activation of cell cycle checkpoint machinery and possible cell cycle arrest. *$p21^{Cip1}$* upregulation was also readily seen in mRNA level, but *$p27^{Kip1}$* mRNA expression was not changed compared to controls (III/Fig. 3 and 4).

To further see the cell cycle progression of AdcD1 infected supporting cells, we decided to study their mitotic activity (III/Fig. 5). We detected only few mitotic supporting cells per utricular explant (4.4 mitotic supporting cells/explant, 0.16% of the total supporting cell population). Next we used another antibody ("PH3-Ser10 broad antibody"), which detects both mitotic cells and cells in late-G2 phase or G2/M boundary (Hendzel et al. 1997, Van Hooser et al. 1998). We found that 11.5% of supporting cells were in late-G2 or G2/M phase. This value is 43.5% of the percentage of Ki-67+ supporting cells. This indicates that cell cycle progression is inefficient and the majority of supporting cells arrest at G2/M boundary. To study the involvement of $p21^{Cip1}$ in the cell cycle arrest, we used utricular explants from *$p21^{Cip1}$* deficient mice. The proliferative activity (number of Ki-67+ cells) in these mutant specimens was comparable to wild types. This indicates that $p21^{Cip1}$ does not have a major role in limiting cell cycle re-entry of AdcD1-infected supporting cells. In contrast, the mitotic activity in *$p21^{Cip1}$*-/- explants was significantly higher compared to wild types (12.8 mitotic supporting cells/explant, 0.47% of the total supporting cell population). However, we also observed high numbers of cells in late-G2 or G2/M phase in *$p21^{Cip1}$*-/- utricles, indicating that $p21^{Cip1}$ is not the sole factor mediating the G2/M arrest.

It has been reported that after *Rb* inactivation early postnatal cochlear supporting cells can go through several rounds in the cell cycle (Yu et al. 2010). Although the majority of AdcD1-infected supporting cells were arrested at the G2/M boundary, a small fraction of them progressed into mitosis. Further, we found rare supporting cells double-positive for the thymidine analogs EdU (pulse given between 3 and 4DIV) and BrdU (pulse given between 7 and 8DIV), suggesting that these cells had completed the cell cycle and continued with another round of replication (III/Fig. 5). However, we cannot exclude the possibility that the EdU+/BrdU+ cells represented G2 arrested supporting cells that underwent endoreduplication, resulting in polyploidy (Niculescu et al. 1998).

Our group has reported the induction of DNA damage in hair cells after forced cell cycle re-entry (I, Sulg et al. 2010). Furthermore, other quiescent cell types, such as cardiomyocytes and myotubes, also respond to cell cycle manipulations with DNA damage (Pajalunga et al. 2010, Campa et al. 2008). To test whether supporting cells with abnormal proliferation also experience DNA damage, we labeled AdcD1 infected explants with p-H2AX antibody, which detects DNA double-strand break foci. We found co-localization of Ki-67 and p-H2AX in several supporting cells, indicating that cell cycle re-entry triggers DNA damage (III/Fig. 6). With high probability, the appearance of DNA damage triggers the activation of DNA damage response leading to cell cycle arrest. Those supporting cells that had progressed to mitosis did not show DNA damage or $p21^{Cip1}$ expression (III/Fig. 6). This indicates that some supporting cells are able to repair DNA damage and progress to mitosis or alternatively, some supporting cells originally experienced only minor DNA damage which allowed them to escape from checkpoint activation.

Cell cycle activation in hair cells ultimately leads to their death. Interestingly, we could not observe apoptosis in cell cycle re-activated supporting cells (III/Fig. 7). As apoptosis is a rapid process, a low-level apoptosis might have been overlooked in our study. Yu et al. (2010) reported apoptosis in early postnatal cochlear supporting cells after *Rb* inactivation. In general, cochlear sensory epithelial cells, especially hair cells, are much more vulnerable compared to the cells of the vestibular sensory epithelia. The same "rule" can possibly be applied to supporting cells to explain why apoptosis was observed in cochlear supporting cells after cell cycle re-entry, but not in vestibular supporting cells. Cell cycle re-activated hair cells very faithfully sustain their differentiated status (I, Mantela et al. 2005, Sulg et al. 2010). In contrast, we found that the expression of certain lineage-specific transcription factors change in cell cycle re-activated supporting cells. Sox9, an HMG-box transcription factor, is specifically expressed in supporting cells. We found that Sox9 expression was downregulated from mitotic supporting cells (III/Fig. 7). Interestingly, in contrast to Sox9, expression of Sox2 was maintained in mitotic supporting cells (III/Fig. 7). These results show that progression of supporting cells into mitosis is associated with selective changes in their transcriptional program. Whether these changes are a prerequisite for progression into mitosis or whether they are related to the replicative stress these cells experience is not clear. In certain other systems, like in chondrocytes, Sox9 has been implicated to be a survival factor (Ikegami et al. 2011). Thus, Sox9 suppression can indicate an unstable condition of supporting cells forced to cell cycle. Based on our results, we can only speculate about the eventual fate of G2/M arrested utricular supporting cells, as we were not able to follow the cell cycle progression of a single supporting cell.

It has been shown in several studies in different experimental set-ups that early postnatal supporting cells have greater proliferative potential compared to mature supporting cells (Gu et al. 2007). We compared whether early postnatal (P9) supporting cells respond differently to ectopic cD1 expression. We found that the response of P9 explants to ectopic cD1 expression largely corresponded to the response of adult specimens (III/Fig. 8). The only clear difference seen between these age groups was that the mitotic activity was significantly higher in the early postnatal supporting cells (12 mitotic supporting cells/explant) (III/Fig. 9).

Most of the earlier studies on cell cycle regulation in inner ear supporting cells have analyzed immature, rather than fully differentiated cells. Furthermore, prior studies on the proliferative capacity of supporting cells have largely focused on the initial events of the cell cycle. Our study focused on adult supporting cells and their capacity to progress through later phases of the cell cycle. The most important finding of our study was the failure of the majority of mature supporting cells to traverse the G2/M boundary in response to ectopic cD1 expression. It has been reported earlier that ectopic cD1 expression is sufficient to promote proliferation of quiescent hepatocytes in adult mice (Nelsen et al. 2001), showing that deregulated cD1 expression itself is not the primary cause of the G2/M arrest that we observed in supporting cells. We observed that ectopic cD1 was expressed both in the nucleus and in the cytoplasm of infected supporting cells. Thus, the partially impaired translocation of cD1 into the nucleus may impair the mitogen responsiveness of utricular supporting cells. Exogenous cD1 has also been shown to accumulate in the cytoplasm of quiescent cardiomyocytes and neurons, leading to impaired phosphorylation of nuclear pRb, and thus, to impaired proliferation (Tamamori-Adachi et al. 2003, Sumrejkanchanakij et al. 2003,

Campa et al. 2008). Taken together, there are several obstacles in the cell cycle progression of mature supporting cells. These obstacles need to be overcome before the proliferative potential of these cells can be used in regenerative approaches.

Figure 5 presents a schematic summary of the results in papers I, II and III.

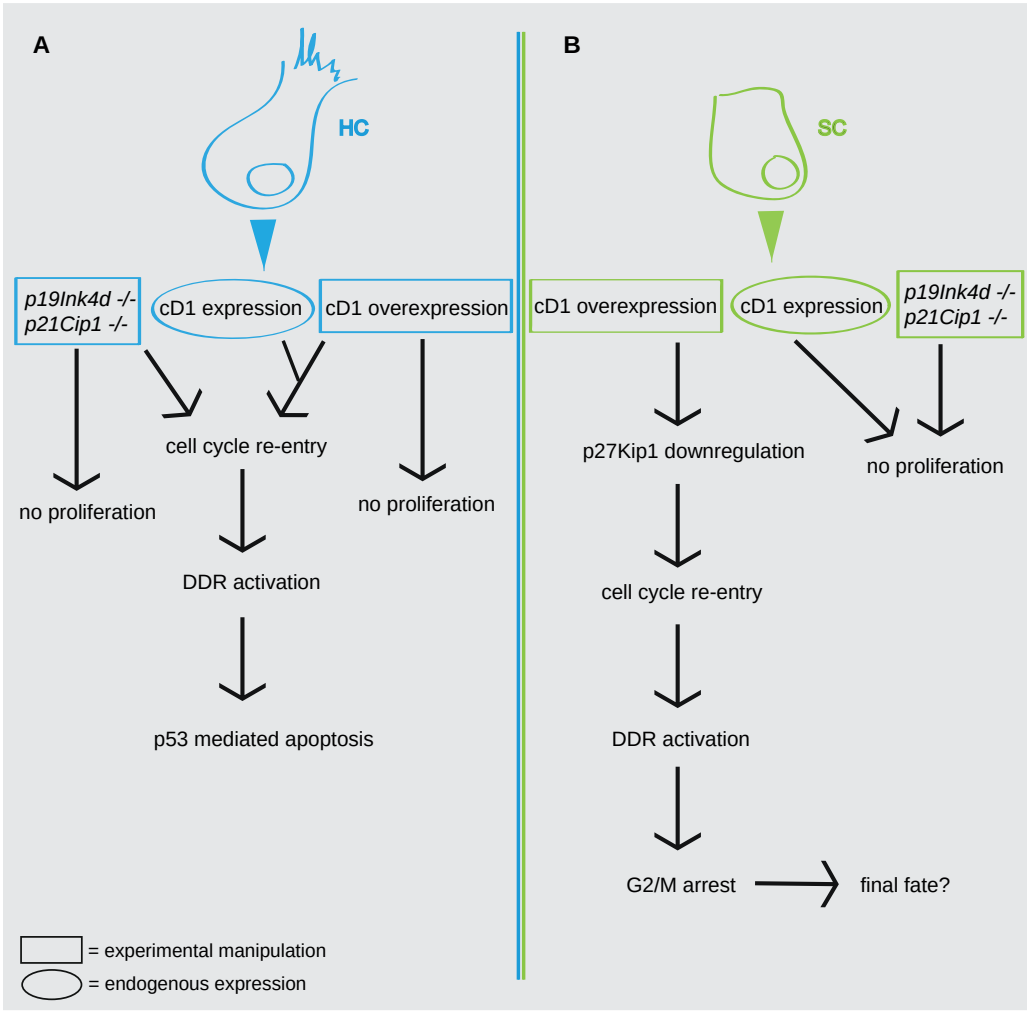

Figure 5. Schematic summary of the results. This schematic view summarises the key findings in papers I, II and III. (A) Cell cycle re-entry in hair cells (HC, blue) is triggered only when $p19^{Ink4d}$ and $p21^{Cip1}$ are inactivated and cyclin D1 (cD1) is expressed simultaneously (either endogenously or ectopically) (I and II). Cell cycle re-entry rapidly leads to the activation of DNA damage response (DDR) and apoptosis. (B) When $p19^{Ink4d}$ and $p21^{Cip1}$ are inactivated, supporting cells (SC, green) do not re-enter the cell cycle, even if cD1 is endogenously expressed in supporting cells (I). When ectopic cD1 is induced in supporting cells they are able to downregulate $p27^{Kip1}$ expression and can re-enter the cell cycle (III). DDR activation is also observed in cell cycle re-activated supporting cells, but instead of immediate death they arrest at the G2/M boundary.

6. CONCLUDING REMARKS

In order to achieve regeneration in the mammalian inner ear, the basic mechanisms that control the maintenance of the postmitotic state in the inner ear sensory epithelia and the restrictions that limit the proliferative potential of mammalian hair cells and supporting cells must be understood first. This thesis work focused on studying the role of positive and negative cell cycle regulators as controllers of the postmitotic state of postnatal hair cells and supporting cells. In addition, this thesis focused on understanding the role of cD1 as a regulator of the proliferative potential of mature supporting cells.

Maintenance of the postmitotic state of hair cells and supporting cells is an active process cooperatively regulated by several CKIs and cD1. The maintenance of the postmitotic state is critical for the lifelong survival of auditory hair cells, and forced cell cycle re-entry leads to hair cell death. Thus, the stimulation of proliferation of auditory hair cells by manipulating the expressions of cell cycle regulators is unlikely to be a valid approach for hair cell regeneration. These findings are in accordance with previous studies on neurons, and other terminally differentiated and highly specialised cells. Several studies have shown that quiescent cells do not tolerate forced cell cycle activation (Zindy et al. 1999, Cunningham et al. 2002). As terminally differentiated cells generally have poor DNA repair potential, the inability to tolerate cell cycle re-activation probably reflects this characteristic. When postmitotic cells are forced into the cell cycle, the replication machinery creates errors which are corrected improperly or not at all. When cell's DNA is damaged, the DDR pathway is activated. In dividing cells the activation of DDR has two possible outcomes, but in quiescent cells, which are defective in DNA repair, the most probable outcome is cell death. However, it is not yet fully understood how big the differences of DDR in quiescent cells compared to dividing cells are. This is one of the questions that needs to be addressed in the future. As it is well documented that terminally differentiated postmitotic cells, such as neurons (Zindy et al. 1999) or hair cells (Chen et al. 2003, Mantela et al. 2005, Sage et al. 2006, I), are capable of re-entering the cell cycle, the major obstacle on the way to functional regeneration may be the lack of DNA repair. The detailed analysis of the DNA repair abilities of hair cells awaits further studies.

Even if hair cells and supporting cells share common precursors, mature hair cells and supporting cells are very different in their functions and properties. Both auditory and vestibular hair cells are structurally and molecularly highly differentiated. Also different cochlear supporting cell types have specific features. In contrast, vestibular supporting cells are a more homogenous cell population with less specialized characteristics. The sensory epithelia in the avian inner ear, even in the hearing organ basilar papilla, resemble those of the mammalian vestibular organs. These epithelia are composed of hair cells that are surrounded by relatively undifferentiated supporting cells. The inner ear supporting cells of birds, fishes and reptiles can produce daughter cells, an event that together with the capacity for phenotypic conversion into hair cells underlies the natural regenerative capacity (Slattery and Warchol 2010). Furthermore, even neonatal mammalian vestibular supporting cells show proliferative potential in certain experimental settings, i.e. in response to exogenous mitogens (Zheng et al. 1997, Montcouquiol and Corwin 2001a, 2001b, Hume et al. 2003, Gu et al. 2007, Lu and Corwin 2008). The higher capacity for proliferation makes supporting cells more attractive candidates for regenerative studies compared to hair cells.

Unfortunately, the natural proliferative potential of mammalian supporting cells declines rapidly after the neonatal period, making mature supporting cells more difficult targets for regeneration. In addition, the more complex cytoarchitecture of the mammalian cochlea makes the regeneration in the auditory sensory epithelium an even more challenging process. Even if it is not fully understood why mammals have lost the ability for regeneration in the inner ear, it is most probably due to the trade-off situation between the sensitive functional properties achieved with the complex structure of mammalian auditory epithelium and cellular plasticity. Cells with more complex structural and functional properties have less plasticity for proliferation and other processes that would be required for regeneration. To ensure the maintenance of the highly differentiated state, an elaborate network controlling the postmitotic state (and possibly other related features) is utilized. In the avian inner ear, the cell cycle regulatory pathways are not well characterized. Thus, it would be valuable to characterize those cell cycle regulators that are involved in the maintenance of the postmitotic state in the avian inner ear, and how cell cycle regulation is changed upon regeneration.

This thesis work shows that cD1 has a clear role in the regulation of the postmitotic state of hair cells. But an even more interesting finding was that the lack of cD1 expression in mature supporting cells correlates with the lack of their proliferative capability. It has been shown in hepatocytes that ectopically expressed cD1 is sufficient to trigger proliferation of quiescent, differentiated cells in adult mice (Nelsen et al. 2001). We expressed cD1 ectopically in mature vestibular supporting cells and found that it can induce robust cell cycle re-entry in these cells. Unfortunately, we also observed that the cell cycle progression was inefficient, and most of the supporting cells did not progress beyond the G2/M boundary. This indicates that the proliferative potential of adult supporting cells is limited.

Several mammalian cell types and tissues have generally very poor regenerative potential compared to their counterparts in lower vertebrates. Lack of proliferative potential is clearly one reason underlying the failure of regeneration in mammals, but another possible explanation is the lack of cellular plasticity in terms of dedifferentiation and re- or transdifferentiation. In lower vertebrates, regenerating cells first lose their differentiation markers (dedifferentiate), re-enter the cell cycle, proliferate, and then differentiate again towards their original fate (redifferentiation) or towards new fate (transdifferentiation). Regeneration in the avian inner ear has been studied for decades, but it is not still fully understood how regeneration takes place at the molecular level. It is known that in the avian supporting cells the expression of Atoh1 occurs rapidly after hair cell damage (Cafaro et al. 2007, Daudet et al. 2009). In addition, Notch pathway components are also upregulated very soon after hair cell damage (Daudet et al. 2009). The study by Daudet at al. (2009) also showed that Notch-mediated lateral inhibition is necessary for defining the fates of hair cells and supporting cells after hair cell damage. Thus, it seems that regeneration generally follows the normal pattern of development. The same signaling regulators and transcription factors are employed during regeneration as are used to regulate the production of hair cells and supporting cells during embryonic development. The detailed understanding of the steps that take place during regeneration in the avian inner ear would be beneficial when designing strategies to induce regeneration in the mammalian inner ear.

In contrast to hair cells that rapidly undergo cell death following cell cycle re-entry, we could not observe supporting cell death in cell cycle re-activated adult vestibular supporting cells. Instead, we observed some changes in the expression of lineage specific transcription factors. Mature supporting cells normally express two Sox-family transcription factors, Sox9 and Sox2. Sox9 became downregulated in those supporting cells that entered mitosis, but the expression of Sox2 persisted. In the developing inner ear, as in many other organ systems, Sox2 is one of the earliest required factors (Kiernan et al. 2005a). Sox2 is required to specify the sensory identity already in the early otic vesicle, and later it is maintained in the supporting cells, but downregulated from hair cells. Thus, the downregulation of Sox9 and persistence of Sox2 expression in mitotic supporting cells may reflect a more progenitor-like identity of these cells. We can only speculate about the final fate of these mitotic supporting cells, as we were not able to follow individual cells in our experimental setup. In many regenerating systems dedifferentiation is often a prerequisite for successful proliferation and subsequent regeneration. For example, in primary differentiated mammalian muscle cells, inactivation of *Rb* is not sufficient to reverse differentiation and to enable cell cycle re-entry (Pajcini et al. 2010). This suggests that muscle cell differentiation is ensured by several mechanisms, which is likely to be the case also in other tissues. According to the study by Pajcini et al. (2010), only concomitant inactivation of Arf ($p16^{Ink4a}$ alternative reading frame) and Rb led to mammalian muscle cell cycle re-entry, loss of differentiation properties, and upregulation of cytokinetic machinery. These results show that inactivation of negative cell cycle regulators is not sufficient to drive dedifferentiation. Thus, possibly also in the inner ear, additional manipulations, in addition to cell cycle activation, are needed to achieve efficient regeneration involving dedifferentiation.

A recent study by Oesterle et al. (2011) showed that inactivation of $p27^{Kip1}$ in adult mice induced the cell cycle re-entry of a limited amount of cochlear supporting cells. However, no new hair cells were generated. This shows that, unlike non-mammalian species, mature mammalian cochlear supporting cells do not have the capacity to naturally transdifferentiate into hair cells *in vivo*. It is not clear why the transdifferentiation capacity is lost. During normal development, cell cycle exit precedes the differentiation of hair cells and supporting cells. In the experimental setups, where supporting cells are forced to proliferate, cell cycle deregulation, i.e. by inactivation of $p27^{Kip1}$, is a permanent event. One possibility is that persistent cell cycle activity inhibits transdifferentiation processes in dividing cells. Thus, to induce transdifferentiation, more transient cell cycle activation strategies may be needed. The importance of transient cell cycle activation was demonstrated in study by Pajcini et al. (2010), where it was shown that only transient suppression of Arf and Rb yielded myoblast colonies that retained the ability to redifferentiate and fuse into myofibers upon transplantation *in vivo.* In the case of the inner ear, an alternative way to induce transdifferentiation could be inducing Atoh1, or other hair cell specific factors, into the newly formed cells.

It is clear that there are still several obstacles and questions that need to be addressed before regeneration in the mammalian inner ear is achieved. The first obstacle is the inefficient cell cycle progression in adult supporting cells. Is the manipulation of DDR the key to achieve completion of the cell cycle? Or is dedifferentiation necessary for cell cycle progression? The second obstacle is clearly the inability of mature mammalian supporting cells to transdifferentiate. The complex

cytoarchitecture of the mammalian cochlea creates another challenge. To preserve the correct function of the hearing organ, the delicate cellular arrangement of hair cells and supporting cells needs to be preserved. In conclusion, this thesis work has provided important information regarding the regulation of the postmitotic state and proliferative potential of different inner ear cell types, and revealed some restrictions that underlie the defective regenerative capacity of mammalian hair cells and supporting cells. This work emphasizes the complexity related to the regulation of the postmitotic state of quiescent cells. Importantly, this complexity creates a challenge, not a barrier, for regeneration.

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